LIPASE CATALYSED HYDROLYSIS OF NON-CONVENTIONAL OIL RESOURCES : KINETICS & OPTIMIZATION STUDY

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Lipase catalysed Hydrolysis of Non-conventional oil resources: Kinetics & Optimization study

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Certificate

This is to certify that the thesis entitled '**Lipase catalysed Hydrolysis of Non-conventional oil resources: Kinetics & Optimization study'** submitted by **Meenakshee Pandey** is a record of an original research work carried out by her under my supervision and guidance in partial fulfilment of the requirements for the award of the degree of **Master of Technology by research** in **Chemical Engineering** during the session July'2011 – July'2013 in the Department of Chemical Engineering, National Institute of Technology, Rourkela. Neither this thesis nor any part of it has been submitted for the degree or academic award elsewhere.

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Dedicated To

My Parents...

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a_v	vacant interfacial site, m^{-1}
β_o	intercept in quadratic regression equation
$\beta_{1,2,3,4}$	regression coefficient for first order terms of variables in quadratic regression equation
$\beta_{11,22,33,44}$	regression coefficients for second order terms of variables in quadratic regression equation
$\beta_{12,13,14,23,24,34}$	regression coefficient for first order interaction terms of variables in quadratic regression equations
D	desirability
E	free enzyme, mol m^{-3}
E*	Interfacial Enzyme (mol/interfacial area), mol m^{-2}
E^*_T	total interfacial enzyme, mol m^{-3}
E*S	enzyme- substrate complex, mol m^{-2}
k_a	adsorption rate constant, m^2 min^{-1}
k_d	desorption rate constant, min^{-1}
k_1	rate constant in forward step of formation of enzyme-substrate complex, m^3 $(mol.min)^{-1}$
k_{-1}	rate constant in backward step of formation of enzyme – substrate complex, min^{-1}
k_2	rate constant in final step of reaction or step of product formation, min^{-1}

K'	apparent rate constant, min^{-1}
K_A	equilibrium constant in interfacial adsorption of enzyme, mol m^{-3}
k_M	constant similar to Michaelis-Menten constant, mol m^{-3}
r	residual
S	Substrate, mol m^{-3}
t	time, minute
v	rate of reaction, mol m^{-3}.min
V_m	maximum velocity, mol min^{-1}
x_i	coded value of the i^{th} variable used in quadratic regression equations
X_i	uncoded value of the i^{th} variable used in quadratic regression equations
X_0	uncoded value of the i^{th} variable at the centre points used in quadratic regression equations
∂X	step change value of the i^{th} variable used in quadratic regression equations

List of Abbreviations

EFAs	**E**ssential **F**atty **A**cids
PSO	**P**umpkin **S**eed **O**il
TSO	**T**obacco **S**eed **O**il
EC	**E**nzyme **C**ommission
IUPAC	**I**nternational **U**nion of **P**ure and **A**pplied **C**hemistry
FFA	**F**ree **F**atty **A**cid
CLA	**C**onjugated **L**inoleic **A**cid
UIC	**U**rea **I**nclusion **C**omplexes
ALA	**α- L**inoleic **A**cid
FTIR	**F**ourier **T**ransform **I**nfrared **S**pectroscopy
GC	**G**as **C**hromatography
GC-MS	**G**as **C**hromatography **M**ass **S**pectrometry
HPLC	**H**igh **P**erformance/ **P**ressure **L**iquid **C**hromatography
OVAT	**O**ne **V**ariable **A**t a **T**ime approach
DoE	**D**esign of **E**xperiments
CCRD	**C**entral **C**omposite **R**otatable **D**esign
RSM	**R**esponse **S**urface **M**ethodology
ANOVA	**Ana**lysis **o**f **Va**riance

Abstract

Essential fatty acids (EFAs) from non-edible oil resources have been in light for last two decades due to their propitious effects in the prevention of diseases and human nutrition. Omega-6 fatty acids like linoleic acid, in the leaves, seeds and flowers of tobacco and in the seeds of pumpkin seed oil have been produced by chemical methods for their use in various purposes. Now-a-days enzymatic methods have been emphasised in industries and R & D sectors for oil hydrolysis to produce fatty acids to fulfil day-by-day increasing demands and to promote environment friendly methods. Lipases, an enzyme that are popularly being used for catalysing oil hydrolysis have replaced chemical methods. Enzymes like lipase from *Candida rugosa,* because of its structural individuality, inhabit non-specificity towards a wide range of fatty acids of different chain length are being utilized for this purpose. These useful fatty acids can fulfil the increasing demands as well as can be utilized in oleochemical industries and other industries like cosmetics, as food supplements in nutrition, in manufacture of paints and varnishes etc.

In this work, focus has been made on existing method of hydrolysis of some non-edible oil resources like pumpkin and tobacco seed oil and possibility of enzymatic hydrolysis of the same to produce linoleic acid. Optimization of process variables for the production of fatty acids from lipase catalysed hydrolysis of pumpkin and tobacco seed oil has been explored in this study.

In lipase catalysed hydrolysis of pumpkin seed oil, temperature, buffer and enzyme concentration has been found to be significant by Response Surface Methodology (RSM) while temperature causes considerable effect in linoleic acid production with speed of agitation having no significant effect. The key mutual interaction has been observed for temperature with pH & enzyme concentration and pH & enzyme concentration.

In lipase catalysed hydrolysis of tobacco seed oil, temperature, enzyme and buffer concentration have imperative effect on hydrolysis rendering to RSM. A strong mutual interaction has been observed for temperature with enzyme concentration and buffer concentration, between pH & enzyme concentration and buffer & enzyme concentration.

Kinetics study shows that the hydrolysis of pumpkin and tobacco seed oil follow first order rate equation with respect to substrate concentration.

Keywords: Non-edible oil resources, hydrolysis, Essential fatty acids (EFAs), pumpkin seed oil, tobacco seed oil, linoleic acid, *Candida rugosa* Lipase, Response Surface Methodology (RSM).

Contents

Chapter 1

Introduction

Chapter 1

Introduction

Abstract

This chapter describes an overview of the present research and its importance. It includes introduction to conventional processes used in industries and laboratories for the hydrolysis of conventional as well as non-conventional oil resources, advantages of enzymatic hydrolysis over chemical methods and the objectives of this study.

1.1 Overview

Increasing consumption of natural resources has depleted their amount to such a level which has compelled researchers and scientists to think novel methods of their production to meet the increasing demands. Vegetable oils are vital to fulfil worldwide nutritional demands and thus are utilized for many purposes in food industries [Idouraine et al., 1996]. Despite the wide range of sources for vegetable oils, the global consumption is governed by soybean, palm, rapeseed, and sunflower oils with 31.6, 30.5, 15.5, and 8.6 million tons consumed per year, respectively [Stevenson et al., 2007]. But these conventional sources of vegetable oil are unable to meet the increasing demands [Idouraine et al., 1996]. Therefore, the need of hour is to survey for other sources to augment the supplies which makes non-conventional oil seeds of much concern to cope this challenge. The need of time is to search for innovative methods and technology which is non-hazardous, renewable and utilizes less energy, so called 'green technology'. Oils from plants i.e. edible and non-edible oils, exemplify a classical source of such 'green' technology which cause no harm to the environment. Fatty acid is the main component of oleochemical industries. Edible oils are being hydrolysed to obtain fatty acids by conventional hydrolysis process for quite a long time. But these processes have a few disadvantages related to them such as release of by-products which are hazardous to environment and consume a large amount of energy. So to prevent such disastrous effects of conventional methodology, scientists have found a new method for oil hydrolysis using biocatalysts like enzymes. Some of the unique properties associated with these enzymes, especially lipase, are selectivity towards substrates and product purity making them advantageous over conventional methods which is quite impossible to attain in latter case.

Essential fatty acids (EFAs) from non-edible oil resources have been in light for last two decades due to their propitious effects in the prevention of diseases and human nutrition. Omega-6 fatty acids like linoleic acid, in the leaves, seeds and flowers of tobacco and in the pumpkin seed oil has been produced by chemical methods for their use in various purposes. Now-a-days enzymatic methods have been emphasised in industries and R & D sectors for oil hydrolysis to produce fatty acids to fulfil day-by-day increasing demands and to promote environment friendly methods. A substantial number of high-value products require fatty acids in their productions. Industrially important fatty acids like essential fatty acids (EFAs) and others like linoleic acid and some other rarely found fatty acids has stepped in various industries for many applications like cosmetics, detergent manufacturing and formulation, food and nutrition, clinical applications, in treatment of deadly diseases like colon and prostate cancer, in making paints and varnishes because they get easily oxidized and after oxidation forms a fixed film on surface. Besides this, some derivatives of these fatty acids are also being used for several purposes including lubricating oils, shampoos and other personal care products.

1.2 Types of hydrolysis

Hydrolysis reaction principally involves reactions of a group of compounds called as triglycerides or fatty esters present in oils and fats, with water to produce valuable free fatty acids and glycerol. There are three major means currently used for the hydrolysis of fats and oils in the assembly of fatty acids, namely, high pressure steam splitting, alkaline hydrolysis and enzymatic hydrolysis. The high temperature and pressure (typically 250-270°C, 70 bar) needed for steam splitting (Colgate–Emery method) makes this practice unsuitable for splitting subtle triglycerides, unconjugated systems which possibly undergo thermal degradation and making hydroxylated fats and oils or polyunsaturated oils to get polymerized. There are also problems associated with alkaline hydrolysis which is cost demanding process and also need to acidify the soaps formed to produce fatty acids products.

1.2.1 Conventional methods of hydrolysis

Oil and lipid hydrolysis is usually carried out in laboratories by conventional methods using chemicals as catalysts [Gunstone F.D., 2004]. Researchers have used acids, base, high temperature and pressure to catalyse oils and fats. Some of the conventional methods for hydrolysis of oil are described below.

1.2.1.1 Base catalysed oil hydrolysis followed by acidification

In this method, oil is usually hydrolysed by water in presence of hot alkali at high temperature (70-100°C). On completion of hydrolysis, acid is gradually added for acidification. Product (fatty acid) from oil produces characteristics colour and odour. The main disadvantage associated with this process is that it requires neutralization of excess mineral acid and disposal of large amount of salts [Johnson and Fritz, 1989].

1.2.1.2 High pressure steam process for oil hydrolysis

This method is used to hydrolyse oil at higher temperature (150-370°C) at reasonably high pressure, and it is then agitated with heated steam. Since the process is energy and cost intensive, and usually associated with unwanted products makes it unsuitable for hydrolysis [Piazza et al., 1991].

1.2.1.3 Twitchell process

This process incorporates heating the sulfuric-acid-washed fat for 20-48 hours in an open tank with steam in a mixture of 25-50% water, 0.5% sulfuric acid and 0.75-1.25% Twitchell reagent [Web Ref. 1]. In a research work [Riegel and Kent, 2003], oil hydrolysed using this method took 6-10 h for complete hydrolysis and it was concluded that higher the molecular weight of oil, slower was the hydrolysis. They further increased the temperature to 260°C to analyse further enhancement in splitting process and observed that the complete process took 8-24 h at which 95% of conversion was achieved. But the main drawbacks accompanied with this process were the thermal denaturation of product and extended reaction time.

1.2.1.4 Colgate-Emery steam splitting process

This is a single step splitting process in which oil and water are fed in a counter-current manner into a continuous flow column at temperature upto 260°C and high pressure usually greater than 60 bars. High operating temperature results in an adequate dissolution of water in oil phase without any mechanical agitation. The oil is fed from the bottom by a high pressure feed pump and water at 40-50% of weight ratio is introduced proximate top of the column. The void volume acts as reaction chamber. Because of injection of high pressure steam helps in raising the temperature to 260°C and the oil goes upward through hot water-glycerol assembling section and passes through oil-water interface where hydrolysis reaction follows. This process is associated with higher conversion usually greater than 96% and in

lesser time but again the major disadvantage is high temperature which leads to thermal denaturation of products [Web Ref. 2; Vulfson, 1994].

1.2.2 Enzymatic hydrolysis

As the demand for products obtained from oil is increasing day-by-day has bound the researches to think over some renewable resources to obtain these useful compounds to meet the increasing demands and maintain the greenery of our mother earth.

It has been known since the commencement of the 20th century that lipases also can catalyse the hydrolysis of fats [Anderson et al., 1998, Compton et al., 2001 and Dossat et al., 2002]. Hydrolysis of vegetable oils using enzymes lipases are being studied and practiced recently to produce fatty acids. Enzymatic hydrolysis utilizes ambient reaction conditions for carrying out hydrolysis making it advantageous over other conventional methods. Use of lipases eases complex downstream processes and leads to reduction in whole operation costs. Splitting of fat with lipase as a catalyst is favourable compared to a conventional route due to greater safety, low energy consumption and high product quality [Linfield et al., 1984 and Kimura et al., 1983]. In enzymatic method of splitting oils and fats, an aqueous solution of lipases is made to contact with oil to form liquid-liquid dispersion. Lipase catalysed esterification reaction has been shown in Fig 1.1.

1.3 Lipases

According to 'Enzyme Commission (EC)' of 'International Union of Pure and Applied Chemistry (IUPAC)' lipases belong to class III enzymes known as Hydrolase and are popularly known as 'triglyceride acylhydrolase' those catalyses the hydrolytic cleavage of C-O, C-N and C-C bonds. Lipases catalyse the splitting of the long chain triglycerides. These nature's catalysts are produced from bio-based materials generally from microbes, animals and plants which constitutes a large part of our earth's biomass. Enzymes are achieving interests in various fields because of their efficiency in accomplishing many novel products in oleochemical industries and in formulation of detergents, in cosmetics industries and many more [Louwrier A., 1998]. These days researchers are in search for rugged products which can be obtained in a sustainable manner i.e., without disturbing the nature's balance and a clean technology, for which lipases have proved themselves as their major impact in various range of applications.

These enzymes are serine hydrolyses which don't require cofactors and catalyses the hydrolytic cleavage [Ghosh et al., 1996]. The catalytic triad is constituted of Ser-Asp/Glu-His and usually also a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site serine. They hydrolyse the triglycerols and emulsified esters at an oil-water interface. Basic reaction catalysed by lipases is the hydrolysis of triglycerides to give di-glycerides, mono-glycerides, free fatty acids and glycerols.

Lipase acts on substrate in specific and non-specific mode, causing in complete or partial hydrolysis of triglycerides into free fatty acids and glycerol or sometimes along with triglycerides, monoacylglycerides and diacylglycerides [Louwrier, 1998]. Specificity by lipases can be shown in two different ways with respect to either fatty acyl or alcohol part of their substrates [Macrae et al., 1985]. They also show both stereopecificity and regiospecificity with respect to alcohol moiety of their substrate [Ghosh et al., 1997]. Again on the basis of regioselectivity, these lipases are categorised into two. First category includes lipase which catalyses the whole breakdown of triacylglyceride to glycerol and free fatty acids along with diacylglycerols and monoacylglycerols which act as intermediates in the reaction, lipase isolated from *Candida cylindracea* provides an instance for such lipase [Kugimiya et al., 1989]. Second group of lipase release fatty acids by acting in regiospecific manner from outer 1 and 3 positions of acylglycerols, hydrolysing triacylglycerol to provide free fatty acids, 1,2- diacylglycerol, 2, 3 and 2-monoacylglycerols, for instance, *Aspergillus niger* [Lawson et al., 1994]. Bezbradica et al. studied the specificity of *Candida rugosa* lipase catalysed esterification reactions in organic media [Bezbradica et al., 2006]. They found that formation of an acyl-enzyme complex is the first step of the reaction and in order to maximize the esters yield, one must need to optimize reaction conditions or parameters like temperature, pH, enzyme conc. etc. In another study accompanied with lipase from *Candida rugosa* in organic media, it was found that lipase from *C. rugosa* has stronger attraction for unsaturated acids. Janssen et al. used same enzyme to study the dependency of the specificity constant (Vm/K_M) on the fatty acid chain length and observed that increasing the number of carbon atoms in the fatty acid caused a constant decrease of initial rate of ester formation [Janssen et al., 1999].Thus, vast diversity of lipases, depending on their abundant properties act on huge range of substrates and many of them are yet to be explored.

1.4 Advantages of Lipases over conventional catalysis

There are several advantages of lipases over conventional catalysts in hydrolysis of oil.

1. Lipases function at or marginally above ambient temperature under atmospheric pressure and a temperate range of pH while conventional or chemical catalysts most often require high temperature or pressure or sometimes both.
2. Due to lower activation energy, lipases hold higher catalytic efficiency which leads to a higher rate of reaction in most of the cases.
3. Lipases are very specific towards its substrate and have higher capability to distinguish among different categories of compounds as compared to conventional catalysts, which accelerates the production of desired product. Besides, side reactions are fewer generating to cleaner products. Therefore, these biocatalysts are preferred for 'Green Chemistry'.
4. Lipases also exhibit substrate specificity, positional specificity and stereo specificity.

1.5 Disadvantages of lipase catalysed hydrolysis over chemical process

The disadvantages of lipase catalysed hydrolysis over chemicals processes are as follows.

1. Low conversion is one of the major disadvantages associated with enzymatic hydrolysis as compared to complex chemical process.
2. High cost of lipases is another obstacle in this field which can be overcome by immobilizing these enzymes for their reuse with almost same efficiency [Rao et al., 1991].
3. In lipase catalysed hydrolysis process, more surface active fatty acid and monoacylglycerol get accumulated at the interface (lipid-water) and replace less active lipase and substrates which ultimately leads to low conversion [Reis et al., 2009].
4. Increasing speed of agitation also affects the overall conversion process because enzymes as well as products get denatured and limits the conversion process [Sadna, 1991].

1.6 Ways to overcome these limitations

The properties of enzymes need to be enhanced for better prospects. They need to be dynamic and adaptable with respect to the array of substrates on which they act without hampering the specificity. Now-a-days immobilization is being practiced over free enzymes, to lessen the high operation cost and to get a better salvage and reusability [Pugazhenthi and Kumar, 2004]. The catalytic latent of lipases can be further improved and made discerning

by the novel phenomena of molecular imprinting and solvent engineering and by molecular approaches like directed evolution and protein engineering [Reetz and Jaeger 1999].

1.7 Limitations of Earlier Studies

There are quite a few studies of lipase catalysed hydrolysis of edible and non-edible oils among which a few studies focused principally on hydrolysis of pumpkin and tobacco seed oil. But efficient optimization of process variables to maximise production of linoleic acid from these oils have not been observed anywhere.

1.8 Objectives of Current study

As discussed above, the processes like hydrolysis of edible and non-edible oil resources to obtain useful fatty acids have been widely studied. But in this work enzymatic hydrolysis of some non-edible oils like tobacco seed oil and pumpkin seed oil has been emphasised to obtain linoleic acid, an essential fatty acid. Also, optimization study has been done. Some specific objectives have been framed as below:

1. Study of lipase catalysed hydrolysis of pumpkin and tobacco seed oil to obtain linoleic acid by single variable optimization method (temperature, pH, buffer concentration, enzyme concentration and speed of agitation).
2. Optimization study of lipase catalysed hydrolysis of pumpkin and tobacco seed oil to enhance the production of linoleic acid by Response Surface Methodology (RSM).
3. Kinetics study of lipase catalysed hydrolysis of pumpkin and tobacco seed oil.

1.9 Scope of the study

This study shall emphasize on hydrolysis of pumpkin and tobacco seed oil using lipase from *Candida rugosa* which is one of the robust lipases, remains non-specific towards an expansive range of fatty acids of diverse chain lengths and illustrates a low activity predominantly towards long, polyunsaturated fatty acids. This enzyme has potential to hydrolyse 16-carbon and 18-carbon fatty acids much faster than long chain fatty acids. Also, kinetics of lipase catalysed oil hydrolysis has been covered in this study.

1.10 Organization of the thesis

In this thesis, it is intended to maximize production of linoleic acid by pumpkin and tobacco seed oil with the help of lipase from *Candida rugosa*. For this reason, effective process variables are optimized in both the cases by conventional single variable method and then by response surface methodology to integrate conjoint effects of process variables.

Chapter 1 is an introductory chapter describing a general overview, types of hydrolysis in brief, a general idea about lipases, advantages & disadvantages of lipases over conventional catalysts and a few points focusing the ways to overcome those limitations. Chapter 2 contains the detailed literature review on various topics related to present work. Chapter 3 includes all the experimental work like materials and methods. Chapter 4 deals with the hydrolysis of pumpkin and tobacco seed oil catalysed by lipase from *Candida rugosa* and effects of single process variables on the hydrolysis of pumpkin and tobacco seed oil respectively. Chapter 5 and Chapter 6 describes the experimental design and optimization study of parameters like temperature, pH, enzyme concentration, buffer concentration on hydrolysis of pumpkin and tobacco seed oil respectively. Chapter 7 explains the kinetic study of lipase catalysed hydrolysis of pumpkin and tobacco seed oil. Chapter 8 contains concluding remarks and future prospective of the present work.

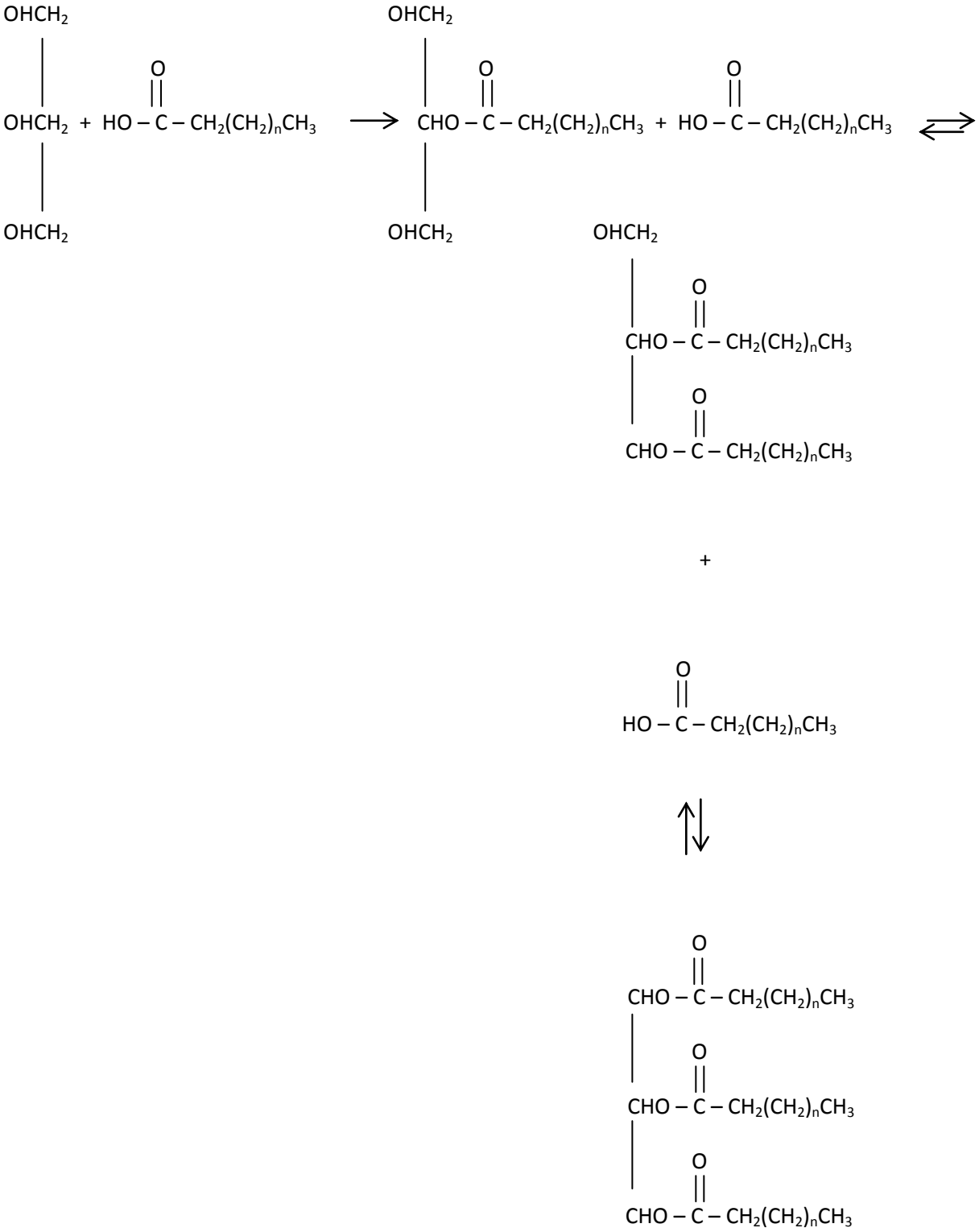


Fig.1.1 Lipase catalysed esterification and hydrolysis of glycerides [Reis et al., 2009**].**

Chapter 2

Literature Review

Chapter 2

Literature Review

Abstract

This chapter deals with the detailed literature review related to the sources of lipases, a brief literature about Candida Rugosa lipase & their action on interface and mainly focusing on enzymatic hydrolysis of oils using different lipases and different methods of conventional hydrolysis that have been used to obtain fatty acids from both edible and non-edible oils.

2.1 Prelude

Researchers have put their continuous efforts for many decades in the field of enzymatic hydrolysis for which many lipases from different sources have been explored and are still going on. Although different lipases have their potential in hydrolysing oil resources, still lipases from yeast *Candida Rugosa* holds its unique place in oleochemical and several other industries for oil hydrolysis because of its diverse advantages over other lipases.

Many research works have already been carried out with edible and conventional oil resources like sunflower oil, rapeseed oil etc. to produce essential fatty acids (EFAs) and other useful fatty acids. Much work is still awaited for examining and characterizing the use of non-edible and non-conventional oil resources like tobacco and pumpkin seed oil which contains useful fatty acids like linoleic acid which is an essential fatty acid besides being an important fatty acid for oleochemical industries. Only chemical methods have been employed till now to carry out hydrolysis of these two oils.

2.2 Sources of Lipases

Lipases are obligatory for lipid hydrolysis which are widely distributed among plants, animals and microbes and are being utilized commercially to serve several industrial purposes. The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases. These lipase have unique properties of acting at lipid-water interfaces which indicates that the kinetics followed by lipase catalysed reactions do not follow Michaelis-Menten equation which generally are suitable for homogenous phase reactions [Aravindan et al., 2007]. There are several other

sources to obtain useful lipases which include plants, animals and microbial too which have been mentioned in Table 2.1.

2.3 *Candida rugosa* Lipase: An Emanate and Resilient Biocatalyst

Candia rugosa is a yeast which is non-sporogenic, pseudofilamentous, non-pathogenic (i.e., not causing disease) and unicellular. In the field of hydrolase based biocatalysis, first lipase was extracted by isolating yeast *Candida Rugosa* from natural soil samples in early sixties and their influential capacities were studied [Yamada et al., 1963 and Tomizuka et al., 1966]. This powerful yeast is capable of producing and secreting mixture of lipase isoenzymes in various proportions. Later, two isoenzymes of same lipase were identified and named as LipA and LipB which were further genetically characterized to explore some useful properties [Longhi et al., 1992 and Lotti et al., 1993].

Lipase *Candida rugosa* has a molecular weight ranging from 57-62 kDa [de Maria et al., 2006a; de Maria et al., 2006b]. Among the five isoforms which have been biochemically characterized, a molecule of this lipase consists of 543 amino acids [Lotti et al., 1993]. This lipase has an ellipsoidal structure having single domain protein built around an 11-stranded mixed P-sheet and of estimated dimensions 66×55×45 $Å^3$. The connections on the N-terminal side of the sheet are rather short, whereas the connections on the C-terminal side are rather long, containing a total of 17 helices and forming a cap on the top of the P-sheet. Now-a-days, among seven genes (Lip 1- Lip7) of *Candida rugosa* which are actually responsible for whole machinery, five have been biochemically characterized (Lip 1-5) [Lotti and Alberghina, 1996]. Initially used names LipA & LipB, have been fully abandoned and new nomenclature has been done as from Lip 1 to Lip 7. Lipase *Candida rugosa* shows a more intricate substrate-binding mode than other lipases due to uncommon shapes of its binding site. In case of *Candida rugosa* lipase (CRL) the polypeptide chain folds over the binding site forming a deep tunnel piercing towards the center of the molecule while in other lipases the sessile acyl chain lies on the surface of the protein [Ghosh et al., 1995 and Grochulski et al., 1994b]. This **tunnel** observed in CRL is unique among the lipases studied to date. This tunnel is 22 Å long with a diameter of about 4 Å (a total size of 25 Å) [Pleiss et al., 1998]. This L-shaped tunnel in CRL starts near active-site Ser-209 residue and can harbour at least 20 carbon atoms long fatty acyl chain. The tunnel is lined by the side chains of Pro-246, Met-213, Leu-302, the aliphatic part of Arg-303, Leu-304, Phe-362, Phe-366, and Val-534. The terminal of the tunnel is sealed off by Tyr-361 and Ser-365. In lipases, the protein surface at

the border of this pocket is hydrophobic and is supposed to interact with the hydrophobic substrate interface as the native structure contains no solvent molecules [Grochulski et al. 1994a and Cygler et al., 1999]. Hence, the triacylglycerol molecule must get accommodated by this lipase to get hydrolysed efficiently. Due to this structural speciality, *Candida rugosa* lipase remains non-specific towards an expansive range of fatty acids of diverse chain lengths (showing much faster activity towards 16 and 18-C fatty acids), and displays a low activity mainly towards long, polyunsaturated fatty acids such as those from fish oil triglycerols [Lie et al.,1986]. Candida rugosa lipase is known to possess another unique feature called as '**flap**' which helps in the closing and opening of the tunnel. This was first proposed to comprise amino acid residues from 62-92 [Grochulski et al., 1993]. This flap is amphipathic character positioned on the protein surface (i.e., on the surface of lipase). One side of the flap contains most of the hydrophilic residues and forms a hydrophilic surface which remains exposed to the solvent. The inside face is comprised of hydrophobic residues and interacts with hydrophobic residues surrounding the active site [Grochulski et al., 1993]. There are 12 well-defined water molecules from the first hydration shell that are coordinated by the residues of the flap. The flap moves almost 90° to open a substrate binding tunnel due to a conformational change from a closed to an open form. Out of three proline residues contained within the flap, two are in a ***trans*** conformation and Pro 92, near the C-terminal end of the flap, adopts a ***cis*** conformation thus takes a major role in flap movement during the interfacial activation [Grochulski et al., 1994a].

2.4 Lipid Water Interface: Site for the action of lipase

Interfaces have proved a significant role in biology. Most of the biological actions occur at interfaces rather than in bulk. Many of the natural substrates of the most broadly studied water-soluble lipases, are insoluble in water **[**Stamati et al., 1999**]**. Therefore, in order to acquire a high lipase activity there must be lipid-water interface **[**Van-Tilebeurgh et al., 1993**]**. It has been established that binding of the lipase to the interface is a critical step in biocatalysis **[**Cordle et al., 1998**]**. Lipases have been amended to deal with the biophysical properties of the interfacial microenvironment where their substrates are found. The regular substrates of lipases are long-chain triacylglycerols, which have very low solubility in water; and the reaction is catalysed at the lipid-water interface and not in the bulk fluid having dissolved substrates. This process of improved lipase activity at lipid-water interface is termed as '**Interfacial Activation'** (Fig. 2.1). Lipases are serine hydrolases which act at the lipid-water interface. Occurrence of an amphiphatic flexible flap partially elucidates the

interfacial activation phenomena which come from the **[**Schrag et al., 1997; Jaeger et al., 1998; Jaeger et al., 1999 and Schmid et al., 1998], so-called closed or inactive state shields over the active-site region of the enzyme, circumventing its exposure to the solvent, but in the open or active state makes it available. This is the reason why, in aqueous media lipases display very little activity. The catalytic triad is tranquilled of Ser-Asp/Glu-His and generally also a consent sequence (Gly-x-Ser-x-Gly) is found around the active site serine **[**Nardini et al., 1999**]**. Serine present at their active site has been shown to be bounded in the highly preserved domain and exemplifies the only common feature shared by all fixed lipases which have been sequenced till now **[**Antonian et al., 1988**]**.

One of the major limitations associated with lipase catalysed hydrolysis, is the build-up of more surface active monoacylglycerol and fatty acid i.e., the products in different stages of hydrolysis, replace less surface active lipase and triacylglycerol from oil-water interface which further decreases the interaction between lipase and triglycerol leading to low conversion. The reason can be explained as the lipases are either *Sn*-1, 3 regiospecific or nonregiospecific [Reis et al., 2009].

Triglyceride hydrolysis catalyzed by a *Sn*-1, 3 regiospecific lipase yields *Sn*-2 monoglyceride and fatty acid, while hydrolysis catalysed by a non-regiospecific lipase will give fatty acid and glycerol. Thus, if hydrolysis occurs with a *Sn*-1, 3 regiospecific lipase, a interfacially active reaction product will form that leads to the expulsion of lipase from interface **[**Reis et al., 2009**]**.

2.5 A Review on Conventional Methods of Hydrolysis

Warsito et al. (2011) performed hydrolysis of basil seed oil, *Ocinum basilicum L*. to obtain enriched α-(alpha) linolenic acid (ALA). Fatty acids were fractioned by crystallization followed by crystallization of fatty acid in urea inclusion complexes (UIC) because conventional crystallization method has restrictiveness to produce appropriate fatty acid purity. ALA was obtained by crystallization, using a mixture of methanol solution at -3, -13, -25°C and urea solution (ratio 1:2) at 4, 2, -6 and -8 °C. The ALA percentages were analysed by GC and GC/MS. They found this technique very useful as the formation of complexes for separation of mixture of saturated and unsaturated organic compounds proved to be a beneficial technique [Bist et al., 2007].They concluded that the percentage of ALA resulted from fractional crystallization at -25 °C increased from 65.16 to 91.40%, and attained from UIC (ratio urea to fatty acid 1:2) is 98.8% at 2 °C.

Salimon et al. (2011) studied the effects of different parameters and their optimization in KOH (alkaline) catalysed hydrolysis of *Jatropha curcas* seed oil. Further, characterization was done using Fourier transforms infrared spectroscopy (FTIR), GC and HPLC. The optimum conditions for maximum FFA% were attained at 1.75M of ethanolic KOH concentration, 65°C of reaction temperature and 2.0 h of reaction time. Ethanolic KOH concentration was found to be a significant variable for hydrolysis of *J. curcas* seed oil.

Czauderna et al. (2011) studied hydrolysis of animal tissues and milk to obtain conjugated linoleic acid. Samples were hydrolysed with 1M KOH in methanol and 2M KOH in water at room temperature for 12 h. Hydrolysates were acidified and the free fatty acids were excerpted with dichloromethane and further analysed by high performance liquid chromatography (HPLC). They concluded that sorbic acid as the internal standard can act as a useful marker for direct quantification of underivatized CLA isomers.

Diaz et al. (2011) studied hydrolysis- hydrogenation of soybean oil and tallow. They used nickel catalysts on alumina support during the hydrolysis of soybean oil and tallow. They observed that maximum conversion was achieved in 3 hours, catalysts 25% NiO/Al_2O_3, temperature of 250°C and 270°C at 250 rpm.

Alenezi and colleagues [Alenezi et al.] analysed the use of sub-critical water for a continuous flow hydrolysis of sunflower oil. They observed that sub-critical water can be used as solvent as well as reactant to carry out hydrolysis without employing alkali or acids. A pressure of 100-200 bar and temperature of 270-350°C were used to obtain fatty acids and glycerol as end products. Fatty acid was found to act itself as an acid catalyst that allowed high production. Increasing temperature and residence time were found to be considerably enhancing the rate of hydrolysis. The highest yield up to 90% was obtained at the conditions of 350 ºC, 200 bar, 8 minutes reaction time and 50:50 v/v water-oil ratio.

In a research, group of researchers performed hydrolysis of oil with sub and supercritical water [Russell et al., 1997]. Supercritical water (SC-H_2O) has received much interest as alternatives to organic solvents [Shaw et al., 1991]. Water is capable of solubilizing most nonpolar organic compounds including most hydrocarbons and aromatics starting at 200-250°C and extending to the critical point [Gao, 1993; Connolly, 1966]. Hydrolytic reaction was carried out with soybean, linseed and coconut oil at a temperature range of 250-375°C and the conditions were optimized for the production of fatty acids,

sodium sulphate was added to the water to salt-out the fatty acids, which were further analysed by supercritical fluid chromatography (SFC) and gas chromatographic analysis of methyl esters of fatty acids (GC-FAME) [Chester, 1996]. Hydrolysis occurred rapidly within 15-20 min, yielding 97% of conversion. But major disadvantage associated with super critical water was found that it thermally degrades the reactants and products.

2.6 A Retrospect on Enzymatic Hydrolysis

Kiatsimkul and co-workers (Kiatsimkul et al.) studied the effects of epoxy groups on enzymatic hydrolysis of epoxy soybean oil. Eight lipases were selected and studied in hydrolysis reactions toward selectively removing saturated fatty acids from epoxy soybean oil. The selectivity of *Candida rugosa* increased toward saturated fatty acids. The presence of epoxy moieties increased the lipid-water interface and enhanced the rate of enzymatic hydrolysis reaction. In addition it was also observed that the epoxy functional group affected the enzyme selectivity.

Serri et al. (2008) carried out preliminary studies about the enzymatic hydrolysis of cooking palm oil using *Candida rugosa* lipase for the production of fatty acids in a batch reactor. Parameters such as temperature, pH, enzyme, oil loading and speed of agitation were examined on the yield of fatty acids. A maximum conversion was attained in 90 min at enzyme loading of 7.46 kLU/ml, oil concentration of 0.1 g/ml, pH 7.5, temperature of 45°C and agitation speed of 200 rpm. Also a kinetic model was developed and described based on enzymatic hydrolysis.

In a high pressure reactor, hydrolysis of sunflower oil was carried out with Lipolase 100T (Aspergillus niger lipase) at temperature range 30-50°C, pH 6-8, pressure 200 bar, rotational speed of 600 rpm and at various enzyme concentrations. And it was concluded that after a certain concentration of enzyme (0.0714 g enzyme/mL), concentration of FFA was not increased with a higher lipase concentration in the reaction mixture. An equilibrium conversion of 92% was achieved after 48 h [Mateja et al. 2003]. The amount of FFA was determined by titration [Leitgeb et al. 1990], and the amounts of free linoleic acid were determined by HPLC [Knez et al. 1994].

Hydrolysis of sunflower oil was performed in a stirred tank reactor consisting of a glass cylinder (10 cm in diameter, 12 cm high), with four stainless steel baffles (1 cm wide) and was catalysed by lipases from *Candida cylindracea*. In this reaction, temperature was

held at 37 °C, pH value at 7 and the agitation rate varied, from 200 rpm to 800 rpm, with increments of 200 rpm. It was concluded that on increasing agitation speed kinetics of the reaction enhanced up to a particular value of the rotational speed where the agitation became turbulent. Free fatty acids concentrations were analysed chemically by titration [Albasi et al. 1999].

Phuah et al. (2012) carried out kinetic study on the partial hydrolysis of palm oil catalysed by *Rhizomucor miehei* for the production of diacylglycerol. On the initial reaction velocity of 7.04 mmol/L/min and reaction time of 6 hrs, the optimum conditions were reported to be 10% (w/w) catalyst with 5% (w/w) water content.

In another study, hydrolysis was performed with oil extracted from pumpkin seed by Soxhlet Extractor using hexane as a solvent. Fatty acids were converted into methyl ester according to the ISO procedure [ISO, Method 5509, 1978]. During whole experiment nitrogen atmosphere was maintained. Petroleum ether was used to extract fatty acid methyl esters (FAMEs) and analysed by gas chromatography (GC) equipped with a flame ionization detector and integrator. Linoleic acid (52.69%) was found to be the main fatty acid constituent in oil [Alfawaz, 2004].

Several research works have exploited the use of lipases to carry out hydrolysis which is a recent advancement in oleochemical industries. Enzyme catalysed process provides better prospects as an alternative green route with all its qualities like broad specificity towards substrates, chemo-regio selectivity, their ability to function near interfaces [Bayramoglu et al., 2002; Hayes 2004 and Deng et al 2005] and cost effectiveness, has challenged conventional processes that are expensive as well as harmful for our environment. Immobilized lipases show better activities [Noinville et al., 2002]. Immobilized *Candida rugosa*, an industrially vital enzyme was used to catalyse hydrolysis of olive oil at optimum conditions (pH 8, temperature = 37°C, reaction time = 30 min). It was observed that after reusing this enzyme the reaction time was same which confirmed the effectiveness of immobilized enzyme for industrial applications. Similar study was performed by Ramachandra Murty et al. (2002) with immobilized *Candida cylindracea* used to hydrolyse rice bran oil. It was reported that extent of free fatty acids produced was linearly proportional up to the enzyme loading of 1650 µg/g of bead. Although a decrease in degree of hydrolysis was observed with second and third batch operations with immobilized enzyme.

In another study recently carried out by Goswami and co-workers on enzymatic hydrolysis of castor oil which was performed using *Candida rugosa* lipase in which focus was made over the maximization of the production of ricinoleic acid by response surface methodology (RSM). A 2^4 full factorial central composite design was used to develop the quadratic model that was subsequently optimized and the optimal conditions were as follows: temperature 40°C, pH 7.72, enzyme concentration 5.28 mg/g oil and buffer concentration 1 g/g oil with 65.5% conversion in 6 h [Goswami et al.2009].

Demirkol et al (2006) reported optimization of enzymatic methanolysis of soybean oil by RSM. A three-level, three factor (variable) face-centred cube design was used for the optimization of methanolysis. It was reported that a few independent variables like enzyme load and temperature effected the methanolysis reaction in n-hexane. A good quadratic model was achieved for the methyl ester production by multiple regression and backward elimination. A linear relationship was also detected between the observed and predicted values ($R^2 = 0.9635$).

2.7 A Review on Kinetics study of Lipase Catalysed Hydrolysis of Oils

Bhandari and colleagues [Bhandari et al., 2013] worked on the hydrolysis of Tuna fish oil using lipase from *C.rugosa* in a biphasic solvent system for the production of free fatty acids (FFAs).Various effects of variables like temperature, pH, solvent concentration etc. were studied. 86.5% of hydrolysis was achieved in 24h. Next, kinetic model was developed and fitted using MATLAB. The average value of kinetic constants were predicted as K_M = 4.26 μ moles FFA/ml, K_{i1} = 6.0 X 10^{-6} μmoles FFA/ mg enzyme·h, K_{i2} = 0.042 μmoles FFA/ mg enzyme·h and K_2 = 122.3 μmoles FFA/ mg enzyme.h.

Mateja and co-workers [Mateja et al., 2005] presented a mathematical model for characterizing the Michaelis–Menten type of sunflower oil hydrolysis, catalyzed by Lipolase 100T. Computer modeling of the kinetics was done for the enzymatic hydrolysis of sunflower oil in a high pressure reactor. The model predicted a good agreement between the experimental and calculated values for the concentrations of oleic and linoleic acids at 95 % confidence level. Jurado and colleagues [Jurado et al., 2008] studied the hydrolysis of emulsified tributyrin by *Thermomyces lanuginosus* lipase (TLL) and developed a kinetic model applied to the initial reaction rates as well as to simulate the time course of the hydrolysis reaction at several conditions of temperatures, lipase concentrations and volume

fractions of tributyrin. The kinetic model succeeded in predicting the time course of the reaction and rate of reactions at sufficient enzyme concentration.

Table.2.1. Sources of lipases

S. No.	Sources of lipases	Name	References
1.	**Plant Sources**	Castor bean lipase	Maeshima et al., (1985)
		Rice bran lipase	Aizono et al., (1973)
		Caesalpinia bonducella seeds lipase	Pahoja et al., (2001)
2.	**Animal Lipases**	Homo sapiens pancreatic lipase	Carriere et al., (2000)
		Homo sapiens gastric lipase	Carriere et al., (2000)
		Porcine Pancreatic lipase (PPL)	Jeon et al., (1999)
3.	**Microbial Lipases**		
	a.) Bacterial Lipase	*B. subtilis*	Lesuisse et al., (1993)
		Bacillus thermoleovorans	Rua et al., (1997)
		Bacillus coagulans	El-Shafei et al., (1997)
		P. cepacia	Dunhaupt et al., (1991)
		P. fluorescens	Kojima et al., (1994)
		Pseudomonas aeruginosa	Chartrain et al., (1993) and Shabtai et al., 1992
	b.) Fungal Lipase	**C. rugosa*	Pernas et al., (2001)
		**Candida cylindracea*	Ghosh et al., (1996)
		Rhizomucor miehei	Herrgard et al., (2000)
		Rhizomucor oryzae	Sharma et al., (2001)
		Neurospora crassa	Kundu et al., (1987)
		Aspergillus niger	Namboodiri et al., (2000)

**Indicates Yeast Lipases*

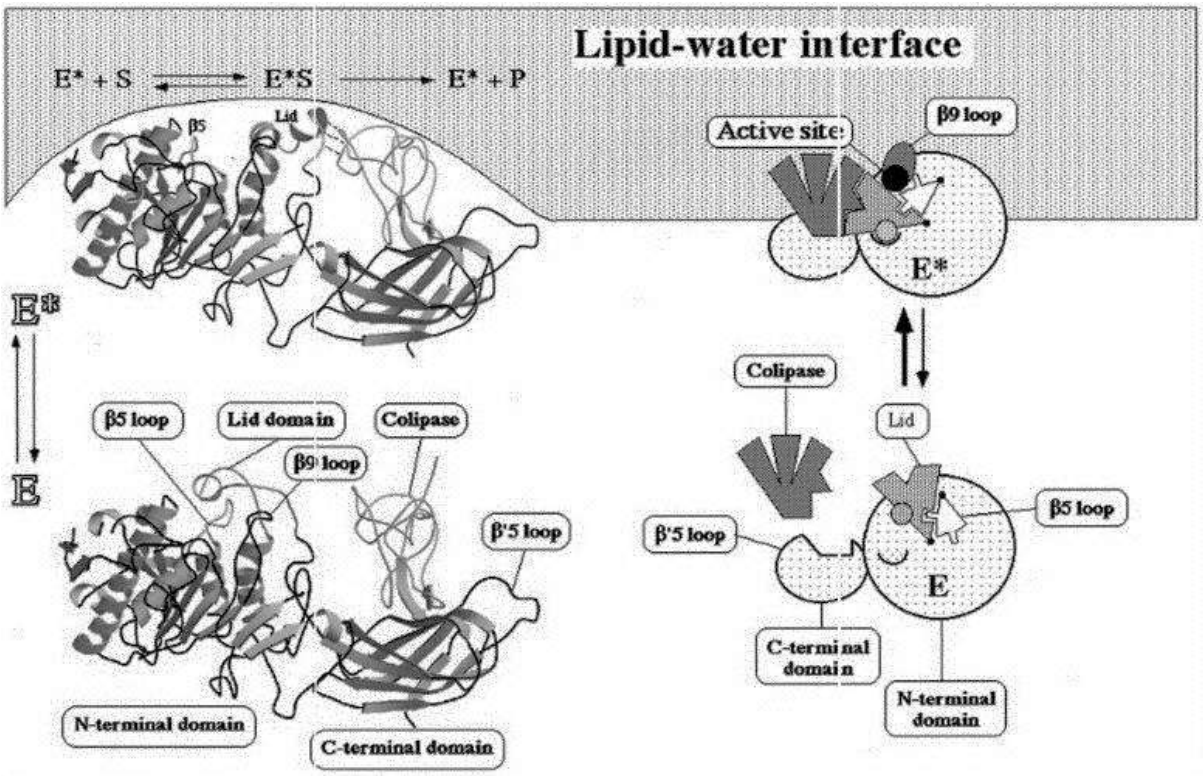


Fig. 2.1 Action of Lipase at Lipid-Water Interface [Van-Tilebeurgh et al., 1993**]**

Chapter 3

Experimental

Chapter 3

Experimental

Abstract

This chapter deals with the detailed experimental procedure followed in all cases of experimental studies. It includes chemicals, equipment details, experimental method and analytical procedure.

3.1 Chemicals

The Candida rugosa lipase (type VII, 965 units/mg solid, were 1 unit activity means production of 1µmole fatty acid) was purchased from Sigma Aldrich Co. India. Pure Linoleic acid (> 99%) was purchased from Sigma Aldrich Co. India. Pumpkin and tobacco seed oil were purchased from Century Oils Ltd. Hyderabad. Analytical reagent grade acetone, toluene and ethanol were obtained from Merck India Ltd. Disodium hydrogen phosphate and sodium dihydrogen phosphate was procured from Merck India Ltd. For analytical purpose, HPLC grade acetonitrile and phosphoric acid were purchased from Merck India Ltd.

3.2 Equipment

All the reactions were carried out in batch mode in a fully baffled mechanically agitated glass reactor of capacity 250 cm^3 (6.5 cm i.d.). A 6.0 cm-diameter four-bladed glass disk turbine impeller with the provision of regulating speed, located at a height of 1.5 cm from the bottom, was used for agitating the reaction mixture. The reactor assembly was kept in a water bath whose temperature could be controlled within ±1ºC. All the experiments were performed under atmospheric pressure. The schematic diagram of the experimental setup is as shown in Fig. 3.1.

3.3 Experimental Methodology

Two approaches were tried while performing experiments. One is conventional, One-Variable-at-a-Time (OVAT) approach (or a single variable approach) where reactions are performed by varying one variable at a time and keeping all other variables fixed. OVAT approach is useful in determining main effect of each parameter on conversion. The second one is Design of Experiment (DoE) where effect of simultaneous change and interaction between two parameters on reaction outcome (percentage of total linoleic acid formed) are

measured. DoE approach is also useful to optimize the process parameters to get maximum conversion and production.

3.3.1 One-Variable-At-A-Time (OVAT) approach

As in most scientific disciplines, chemists have historically followed the practice of changing one variable at a time (OVAT) during optimization [Akhnazarova and Kafarov, 1982; Box et al., 1978]. This method is useful to see the main effect of each variable on conversion and selectivity separately. The data obtained by this method, can be used to get the order of reaction, optimize a simple reaction system, estimate kinetic parameters, to evaluate some thermodynamic parameters. For a complex system, the conventional OVAT approach is time-consuming, non-feasible and inept of getting the true optimum condition due to the lack of interactions among the variables. Also, for systems where mechanism of reaction is not well known, this approach is inadequate for model building and optimization.

3.3.2 Design of Experiments

An alternative approach to OVAT is Design of Experiment. In the DoE approach, process variables are first 'screened' to determine which are important to the outcome. This is followed by 'optimization' where the best settings for the important variables are determined. In particular, factorial designs and response surface methodologies (RSM) have been successfully applied as DoE techniques in both discovery and development. RSM is a statistical technique for developing and optimizing processes, its response (sometimes called objective function) is influenced by several variables [Khuri and Cornell, 1987; Montgomery, 2001; Myres and Montgomery, 2002]. RSM, a combination of some mathematical and statistical procedures, is applied to study relationships between independent variables and response. It uses quantitative data obtained from accurately designed experiments to solve multivariate equations. Response surfaces are the graphical representations of these equations. These surfaces actually explain individual, cumulative, and interactive effects of test variables on response. Further, it determines optimal factorial combination of variables that result in maximum response [Khuri and Cornell, 1987; Montgomery, 2001]. RSM is widely used now-a-days for process optimization through a relatively smaller number of systematic experiments that can reduce time, cost and resources and has the advantage that it allows the user to gather large amounts of information from a small number of experiments. Another advantage of any DoE methods is that it doesn't require any knowledge of detailed mechanism of the reaction system and it just requires the input variables and the responses.

Since 1990s, there are many examples of parallel synthesis and DoE methods which have been applied for process optimization. However, RSM has been used so far as a tool mostly for optimization in biomedical, biochemical, agricultural and pharmaceutical fields with very few applications in chemical fields.

One of the mostly used RSM methods is Central Composite Rotatable Design (CCRD) method. In this method, selection of the levels of variables was carried out on the basis of results obtained in the preliminary study, considering limits for the experimental set-up, working conditions for each chemical species and the previous experiences in dealing with variables to get desired results. For statistical calculations, the variables X_i were coded as x_i according to the following relationship:

$$x_i = \frac{X_i - X_o}{\partial X}$$

where, x_i is dimensionless value of independent variable, X_i represents the real value of independent variables, X_0 is value of the X_i at the centre point and ∂X represents the step change.

The CCRD helps in investigating linear, quadratic, cubic, and cross-product effects of reaction variables on responses. The CCRD permits the response surface to be modeled by fitting a polynomial with the number of experiments equal to $2^f + 2f + n$, where f and n are the number of factors and centre runs, respectively. The center points were used to evaluate the experimental error and the reproducibility of the data. The axial points ($2f$) are chosen such that they allow rotatability, which ensures that the variance of the model prediction is constant at all points and equidistant from the design center. Replicates at the center are very important as they provide an independent estimate of the experimental error.

The statistical software package Design-Expert version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA, was used for multiple regression analysis of experimental data to fit the polynomial equations developed. 3D response surfaces and contour plots were developed using the fitted polynomial equation obtained from regression analysis, holding two of the independent variables at a constant value corresponding to stationary point and changing the other two variables. The quality of fit of the model was evaluated by the coefficient of determination (R^2 and adjusted R^2) and analysis of variance (ANOVA). ANOVA was also used to estimate the statistical parameters. The model was refined after insignificant coefficients were examined and manually eliminated.

3.3.3 Optimization using Desirability Function

The desirability function approach is one of the most widely used methods in industry for dealing with the optimization of multiple response processes. The method is attractive because it is intuitive and simple. The inputs are mean response estimates, target value, and upper and lower acceptability bounds for all factors and responses.

For each response, $Y_i(x)$, a desirability function $d_i(Y_i)$ allots number between 0 and 1 to the apparent values of Y_i, with $d_i(Y_i) = 0$ signifying a completely undesirable value of Y_i and $d_i(Y_i) = 1$ representing a completely desirable value. The individual desirability are then combined using the geometric mean, which provides the *overall desirability D*:

$$D= (d_1\times d_2 \times d_3 \times \ldots. \times d_k)^{1/k} = [\prod_{u=1}^{n} d_u]^{1/n}$$

where *k* denotes the number of responses. Notice that if any response is completely undesirable ($d_i (Y_i) = 0$) then the overall desirability is zero. The desirability function involves transformation of each estimated response variable Y_i to a desirability value d_i, where $0 \leq d_i \leq 1$. The value of d_i increases as the "desirability" of the corresponding response increases.

The optimization module in Design Expert searches for combination of factor levels that simultaneously satisfy the requirements placed on each of the responses and factors. According to the optimization step of the program, the desired goal for each variable and each response should be chosen. The possible goals are: maximize, minimize, target, within range, none (for response only) and set to an exact value (factors only). The goals are then combined into an overall desirability function (*D*). The program seeks to maximize this function.

3.4 Experimental Layout

3.4.1 Procedure for Single Variable Optimization and Multivariate Optimization Process

Firstly, phosphate buffers of different pH were prepared following standard procedure [Gomori, 1955]. For each experiment, 50 ml of pumpkin and tobacco seed oil were added to the glass batch reactor separately for both oils. Temperature of the reactor containing oil was set to the requisite value. 50 ml of phosphate buffer, having appropriate pH, containing specific weight of *Candida rugosa* lipase was then added to the reactor. Next it was agitated at certain speed of agitation with the help of digitally controlled mechanical stirrer for 5 h.

Then 100 ml of neutralized 1:1 (v/v) acetone-ethanol mixture was added to the reaction mixture to inactivate lipase and thus the reaction was terminated.

3.4.1.1 For Pumpkin seed oil

As 1 rpm stands for impeller tip velocity of 0.105 cm/sec, variation of speed of agitation from 600 to 1400 rpm correspond to impeller tip velocity of 63 to 147 cm/sec. After that, pH was changed from 6 to 8 at optimum speed of agitation and other variables kept constant. Next, temperature was varied from 20 to 40º C at optimum speed of agitation, pH and other fixed variables. Then, at optimum speed of agitation, pH and temperature and at fixed lipase concentration, buffer concentration was changed from 1 to 5 g/g oil. At last, lipase concentration was varied from 1 to 13 mg/g oil at optimum speed of agitation, pH, temperature and buffer concentration.

3.4.1.2 For Tobacco seed oil

Similarly, for tobacco seed oil speed of agitation was varied from 500 to 1300 rpm (52.5 cm/sec to 136.5 cm/sec impeller tip velocity). Afterward, pH was changed from 6 to 8 at optimum speed of agitation and other variables were kept fixed. Next, temperature was varied from 20 to 40º C at optimum speed of agitation, pH and other fixed variables. Then, at optimum speed of agitation, pH and temperature and at fixed lipase concentration, buffer concentration was changed from 1 to 5 g/g oil. At last, enzyme concentration was varied from 2 to 18 mg/g oil at optimum speed of agitation, pH, temperature and buffer concentration.

3.4.2 Analysis of Linoleic Acid by HPLC

The resultant mixture was isolated into two layers. Sample collected from upper oily layer was dissolved in absolute ethanol. A 20µl of this solution was injected into an Agilent Zorbax SB C-18 reverse phase HPLC column (4.6 mm inner diameter, 250 mm length and 5µm particle size with controlled pore size of 80 Å). Sample was eluted with 80:20 (v/v) acetonitrile/ 30 mM phosphoric acid at 30ºC at a flow rate of 0.75 ml/min. A UV detector was used in which absorbance was maintained at 205 nm [Bodalo-Santoyo et al., 2005].

3.4.3 Procedure for Kinetics study of lipase catalyzed hydrolysis of oil

At first, buffers of desired pH were prepared using standard procedure [Gomori, 1995]. In each experiment, 50 ml of oil was added to the reactor and heated to the reaction temperature. When the oil reached the desired temperature, a 50 ml of aqueous buffer solution containing lipase was added to the solution such that a certain oil (substrate) and

lipase (enzyme) concentration were maintained. The mixture was stirred for 5 h at a particular agitation speed. The substrate concentration was then varied by taking out sample from the final phase, keeping other parameters constant. Similarly, new sets of reaction were carried out at different enzyme concentrations. Then temperature was varied keeping enzyme concentration at its optimum value. Each reaction was carried out for a comparatively small span of time (20 minutes) to calculate initial velocity.

3.4.4 Analysis for calculation of hydrolysis rate

At certain time interval, reaction was stopped and the reaction mixture was allowed to settle for a while. Certain volume from oil phase was pipetted out and then further separated by a separating funnel into oil and aqueous phase. Sample (2 to 10 ml) was taken from this final oil phase was then added to 50 ml of neutralized ethanol: toluene (1:1, v/v) mixture. Next, it was titrated against standardized ethanolic KOH solution. From the titer value, rate of hydrolysis was calculated.

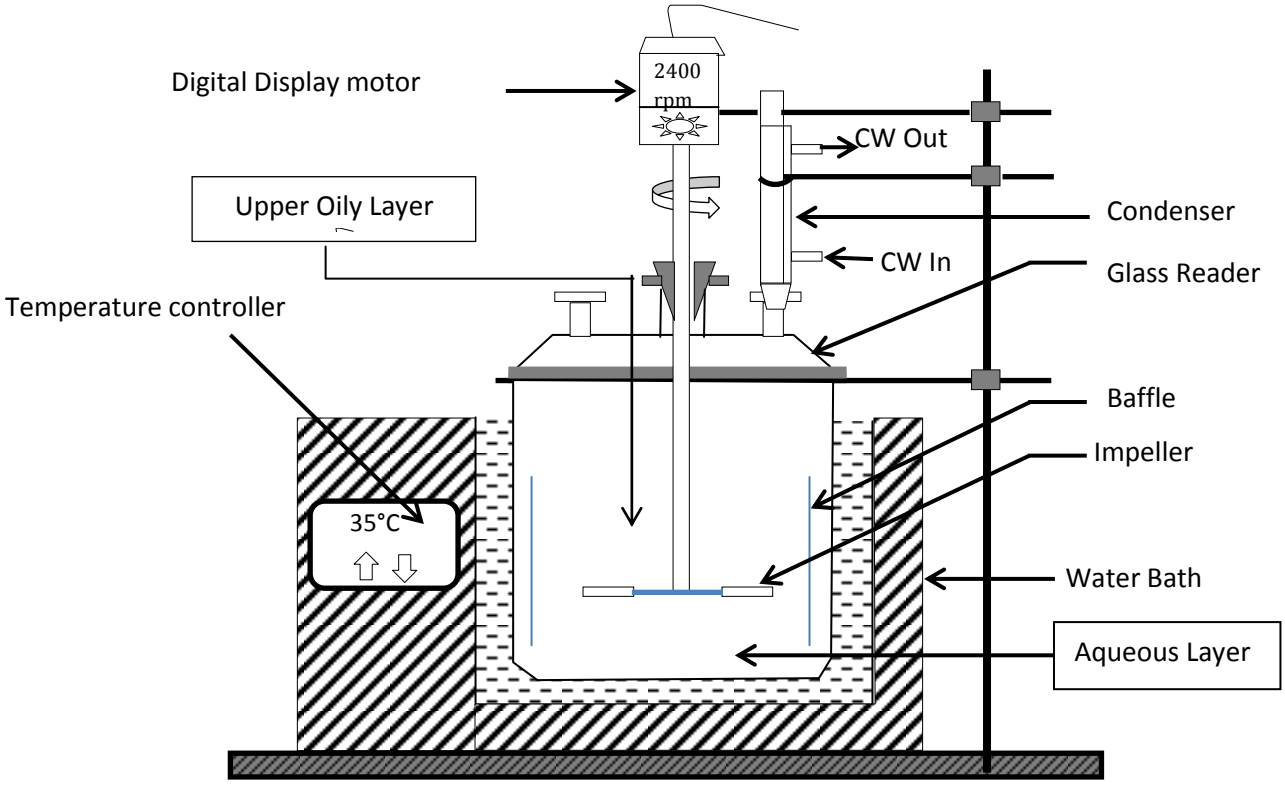


Fig No. 3.1 Schematic Diagram of batch reactor assembly

Chapter 4

Lipase Catalyzed hydrolysis of Pumpkin and Tobacco Seed Oil to Linoleic acid - Single Variable Optimization Method

Chapter 4

Lipase Catalyzed hydrolysis of Pumpkin and Tobacco Seed Oil to Linoleic acid - Single Variable Optimization Method

Abstract

This chapter describes the detailed experimental studies on lipase catalyzed hydrolysis of Pumpkin & Tobacco seed Oil for the production of linoleic acid. In this chapter, single variable optimization approach has been followed to observe the effects of individual parameters like agitation speed, pH, temperature, buffer concentration and enzyme concentration on hydrolysis reaction catalyzed by lipase from Candida rugosa.

4.1 Introduction

Linoleic acid, a 18-carbon fatty acids, is chemically termed as 9, 12 Octadecadienoic acid. It has a hydroxyl group on 18^{th} carbon and two cis double bonds with first double bond located at 6^{th} carbon from methyl end. It is a clear to yellow color liquid having molecular weight 280.45, specific gravity 0.913, boiling point of 227-229 ºC and meting point of 12ºC under atmospheric pressure [Web Ref. 3].

4.1.1 Industrial Applications of linoleic acid

Linoleic acid is a hydroxyl fatty acid which is used in oleochemical industries for several purposes. Linoleic acid is considered as one of the two families of essential fatty acids. Since human body can't synthesize essential fatty acids therefore they are needed to be taken from external dietary sources. Therefore, this particular fatty acid has several applications in pharmaceutical industries for making drugs. Linoleic acid is used in manufacture of quick-drying oils, which are useful in varnishes and oil paints manufacturing industries. These applications impose the easy reaction of the linoleic acid with oxygen in air, which leads to crosslinking and development of a perpetual film. They are also used in making surfactants and dispersants as well as some other synthetics and long chain compounds [Awola et al., 2010].

Reduction of linoleic acid yields linoleyl alcohol. Linoleic acid has become progressively more popular in the beauty products industry because of its favourable properties on the skin. Research also says that linoleic acid has acne reductive, anti-

inflammatory and moisture absorbent properties when applied topically on the skin because of which it is used in cosmetic industries [Web Ref. 4].

4.1.2 Potential of Tobacco seed oil

Tobacco (*Nicotiana tabacum* L.) is one of the chief crops among the major cash crops of India. Tobacco, also known as 'The Golden leaf' is one of the world's top non-food crops. India ranks second in standings of area with 45 lakh hectares and third in terms of production with 700 million kg of Tobacco. Tobacco seeds are a by-product of leaves production of tobacco (*Nicotiana tabacum L.*, family *Solanaceae*). Tobacco seed oil is free from nicotine and is superior to other commercially existing seed oil like cotton oil, groundnut oil etc. [Patel et al., 1998 and Chakraborty et al., 1998]. The miniature tobacco seed contains 35 per cent oil and the oil is predominantly contains linoleic oil, as it is the major fatty acid (66 – 76%) followed by oleic (18.5–28.4%), palmitic (9.1–12.3%) and stearic acid (2.1–3.3%) [Frega et al., 1991; Srbinoska et al., 2003 and Satyanarayana Murthy, 2010]. It has saponification value of 199, iodine value of 135 and acid value 3.20 [Satyanarayana Murthy, 2010]. In India, however, the tobacco seed oil is not being utilized for edible purpose but finds widespread use in lubricants, paints, varnishes and soap manufacturing industries [Awola et al., 2010]. Apart from phytochemicals such as nicotine, solanesol (having anti-ageing, anti-cancer, and anti-diabetic properties), citric acid and malic acid which are present in tobacco some edible protein from green tobacco leaf and oil from the seeds are two areas where additional research could rationalize cultivation of tobacco for different uses [Patel et al., 2012].

4.1.3 Potential of Pumpkin seed oil

Pumpkins belong to the family *Cucurbitaceae*. It. is broadly cultivated all over India for its large fruits with substantial storage size. It is grown in nearly all the states of India. Precise area under pumpkin cultivation in India is not known. A number of studies have stated the chemical composition and oil characteristics of the pumpkin seed differ with origins and diversities [Lazos 1986; Stevenson et al., 2007]. The colour pumpkin seed oil varies from dark green to brown. The pumpkin seeds retain beneficial dietary and medicinal potentials. Pumpkin seed extract has been described to have antitumor, antimutagenic antibacterial, antidiabetic and antioxidant activities [Gossell-Williams et al., 2006 Tsai et al., 2006]. Chemical composition of pumpkin seed oil is wide but the main fatty acid content of this oil is linoleic acid ranging from 49.6- 69.8 %, followed by linolenic (up to 60.8%), oleic

(up to 46.9%), palmitic (up to 14.5%), and stearic (up to 7.4%) acid [Murković et al. 1996; Alfawaz Mohammad 2004; Gilbert et al., 2007 and Fokou1 et al., 2009] It has saponification value of 190.69 ± 1.40, iodine value of 104.36 ± 0.04 and acid value 0.78 ± 0.02 [Gohari Ardabili et al., 2011]. Thus, various studies report that pumpkin seed oil has potential for being used in wide ranging industries like pharmaceutical, oleochemical and cosmetics manufacturing industries etc.

4.2 Results and Discussion

4.2.1 Lipase catalyzed hydrolysis of Pumpkin & Tobacco seed oil:

Lipase is an enzyme which is proteinous in nature. Structure of enzymes are stabilized by weak interaction forces which lead to a functionally important molecular flexibility, therefore it is very important to study effects of various parameters (both physical and chemical) that can possibly change the geometrical and chemical structure of native protein.

4.2.1.1 Effect of Speed of Agitation

Lipase has a distinctive property of acting at the oil-water interface. In order to acquire a high lipase activity there must be lipid-water interface [Van-Tilebeurgh et al., 199]. The substrates of lipases are generally long-chain triacylglycerols, which have very low solubility in water; and the reaction is catalysed at the lipid–water interface and not in the bulk fluid having dissolved substrates. This process of improved lipase activity at lipid-water interface is termed as '**Interfacial Activation'.** There follows a special fit between the respective geometries of lipase active site and substrate groups. This results in high rate of hydrolysis [Verger, 1980]. Therefore, it has been observed and reported that rate of hydrolysis increases with increase in interfacial area and high adsorption of lipase at the interfaces [Sadna, 1991].

Increasing speed of agitation helps in increasing interfacial area and the number of fine droplets in both dispersion medium (buffer) and dispersed phase (oil). This leads to accumulation of high lipase concentration at interface and increases the rate of hydrolysis. But continuous increase in agitation speed leads to enzyme denaturation as it causes unfolding of geometrical structure of active sites of lipases and further causes denaturation. At low speed of agitation, effect of increase in interfacial area predominates over lipase

denaturation. But after a certain speed, denaturation predominates and decreases the rate of hydrolysis [Sadna, 1991].

In case of pumpkin seed oil, agitation speed was varied from 600-1400 rpm while for tobacco seed oil it was studied in range of 500-1300 rpm. The optimum speed of agitation was found to be 1200 rpm (126 cm/sec impeller velocity) in case of pumpkin seed oil hydrolysis (PSO) as can be seen in Fig. 4.1 whereas it was observed to be 1100 rpm (115.5 cm/sec impeller velocity) for tobacco seed oil (TSO) as shown in Fig. 4.2. After optimum speed it was observed that linoleic acid formation decreased probably due to lipase denaturation at very high speed.

4.2.1.2 Effect of pH

pH shows a significant role in lipase catalyzed hydrolysis as it leads to conformational change of lipase by imposing a change of strain on 'lid' covering the active site, i.e., directly affects the opening and closing of catalytic centre for substrate binding. Lipase is very subtle to the operating pH condition because it might also alter the ionization states of the enzyme, which affect its activity and selectivity [Serri et al. 2008].

It has also been reported in a few studies that change in pH affects substrate concentration at the interface. This change leads to ionization of free lipase, free substrate or lipase-substrate complex and change the lipase activity.

At extreme pH, irreversible denaturation of lipase occurs because of breakdown of substrates causing lowering of substrate concentration and also breakdown of products which acts as an inhibitor of lipase, ultimately leading towards the low rate of hydrolysis [Tipton and Dixon, 1979; Verger et al., 1973]. At optimal pH, lipase is most vigorous due to largest share of catalytic active site in total enzyme [Kuo and Gardner, 2002] leading to maximum product formation.

Therefore, a pH in range of 6 to 8 was chosen for both pumpkin and tobacco seed oil to study the effect of the same on lipase catalyzed hydrolysis. Fig 4.3 and Fig 4.4 represents the effect of change of pH of buffer solution on 'percentage of total linoleic acid formed' on lipase catalyzed hydrolysis of pumpkin and tobacco seed oil.

From both figures it can be depicted that conversion to linoleic acid is first increasing with increasing pH and then maximum followed by decrease in conversion. An optimum pH of 7.5 and 7.0 was observed in case of PSO and TSO respectively.

4.2.1.3 Effect of Temperature

The production of total linoleic acid produced from the hydrolysis of pumpkin and tobacco seed oil using *C. rugosa* lipase was also studied as a function of temperature (Fig. 4.5). Temperature may affect the hydrolysis reaction in a positive way or vice versa. An increase in temperature will increase the reaction rate as explicated by the transition state theory. On the other hand, at a higher reaction temperature, enzyme tertiary structure may disrupt leading to denaturation [Romero et al. 2005]. Temperature has its effects on ionization state of enzyme, solubility of substrate in solution. Simultaneous formation of interfacial area and emulsion also depends on temperature of reaction medium.

Thus, temperature imposes two kinds of effects on rate of hydrolysis either by increasing interfacial activation of lipases or by thermal denaturation of lipases [Laidler and Peterman, 1979]. At higher temperature atoms in lipase molecules gain energies and tend to move with greater tendency causing an increased rate of hydrolysis. Also, enough thermal energy is produced to break some of the intermolecular interactions between polar as well as the hydrophobic forces between the non-polar groups within the enzyme structure causing denaturation of enzyme. [Romero et al, 2005]. Subsequently, overall rate of reaction declines.

In this study, temperature was varied from 20ºC to 40ºC in case of both pumpkin and tobacco seed oil. Temperature at which maximum conversion to linoleic acid occurs is known as optimum temperature. So, the optimum temperature was observed to be 35ºC in both cases (both PSO & TSO) as given in Fig 4.5 and 4.6.

4.2.1.4 Effect of Buffer Concentration

In lipase catalyzed hydrolysis, buffer concentration plays a vital role as it directly influences the interfacial area where action of lipase takes place. The quantity of buffer salt present during lyophilization (while doing pH alteration of the enzyme) increases reaction rates simultaneously leading to the formation and accrual of products. It is supposed that the improved activity with increasing buffer content results from a better dispersion of the enzyme in the reaction medium which further prevents intermolecular interactions resulting into mass-transfer limitations and changes in the enzyme conformation [Triantafyllou et al.

1997; Ru et al. 2000 and Straathof, 2003]. Mass transfer limitation in aqueous phase results in quite high substrate concentration at the interface and increased adsorption of lipase at interface leads to its higher concentration at interface than bulk concentration and surges lipase stability [Straathof, 2003].

But ultimately, lipase activity is unfavorably affected by two effects. One is, during hydrolysis, accumulation of fatty acids, monoacylglycerol and diacylglycerol follows at interface. These compounds show higher attraction towards interface than lipase and contend to occupy the interface and replace it [Reis et al, 2009].This further result in decreased contact between triacylglycerol (product) and lipase. Henceforth, rate of hydrolysis is slowed down.

Another reason for decreased rate of hydrolysis is the formation of high concentration of free or ionized carboxylic acid groups from free fatty acids. These groups acidify microaqueous phase neighboring the lipase and results in desorption of water from interface. Also presence of glycerol and low water in reaction zone leads to backward reaction because water is an essential element for high conversion [Kuo and Gardner, 2002].

Fig 4.7 and 4.8 has shown the effect of buffer on lipase catalyzed hydrolysis of pumpkin and tobacco seed oil which was varied from 1 to 5 g /g oil. Optimum buffer concentration was found to be 1 g/g oil for both pumpkin and tobacco seed oil.

4.2.1.5 Effect of Enzyme Concentration

Enzyme concentration has a huge effect on the rate of hydrolysis. Higher the enzyme concentration, higher is the adsorption of lipase in interface from bulk and higher will be the binding with substrate leading to higher conversion to products [Straathof, 2003]. When interface is accommodated with lipase molecules, further increase in lipase concentration has no effect on the rate of hydrolysis as all enzyme molecules gets saturated with substrate molecules.

Fig. 4.9 and 4.10 depicts the effect of enzyme concentration on ‘percentage of total linoleic acid formed’. In case of pumpkin seed oil enzyme concentration was varied in range of 1 to 13 mg/g oil and 2 to 18 mg/g oil for tobacco seed oil. Optimum enzyme concentration was found to be at 10 mg/g oil and 14 mg/g oil for pumpkin and tobacco seed oil hydrolysis respectively.

4.3 Conclusions

One variable at a time approach (OVAT) leads to 1200 rpm and 1100 rpm as optimum speed of agitation for pumpkin and tobacco seed oil respectively. Optimum pH was observed to be 7.5 and 7.0 in case of pumpkin and tobacco seed oil respectively. Before and after optimum pH, production of linoleic acid was attained quite low. Optimum temperature was noted down as 35°C in both cases of oil hydrolysis after that the hydrolysis decreased probably because of denaturation of lipase *Candida rugosa* at very high temperature. Buffer concentration was perceived to be optimum at 1 g/g oil for both oil samples. The reason can be explained as, at 1 g/g oil buffer concentration there is maximum production of linoleic acid in case of pumpkin seed oil and at higher concentration it decreased to a larger extent because of mass transfer limitation or probably because of the presence of inhibitors of lipase. Similarly, for tobacco seed oil although the production of linoleic acid was found to be almost equal at 1 and 2 g/g oil of buffer concentration yet 1 g/g oil was chosen as optimum because it required a less amount of buffer for maximum production of linoleic acid. The degree of hydrolysis was examined to get amplified with an increase in the amount of lipase until it reached plateau. To increase the rate of hydrolysis, it is obligatory to add consecutively high amounts of lipase [McNeill et al. 1996]. Optimum value of enzyme concentration was monitored at 10 mg/g oil and 14 mg/g oil.

Table No. 4.1 Variation in linoleic acid content of different oils [Web Ref. 5**]**

Types of oil	Linoleic acid content (%)
Safflower oil	78
Sunflower oil	68
Pumpkin seed oil	62.8
Hemp oil	60
Cottonseed oil	54
Soybean oil	51
Sesame oil	45
Rice bran oil	39
Canola oil	21
Linseed oil	15
Olive oil	10
Palm oil	10
Coconut oil	2

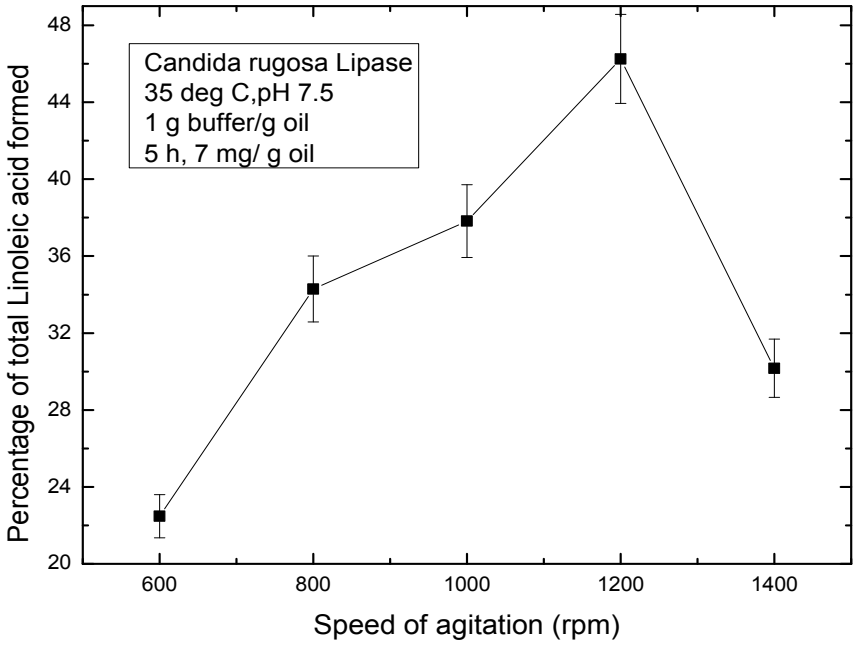


Fig. 4.1 Effect of Agitation speed on 'percentage of total linoleic acid formed' (PSO)

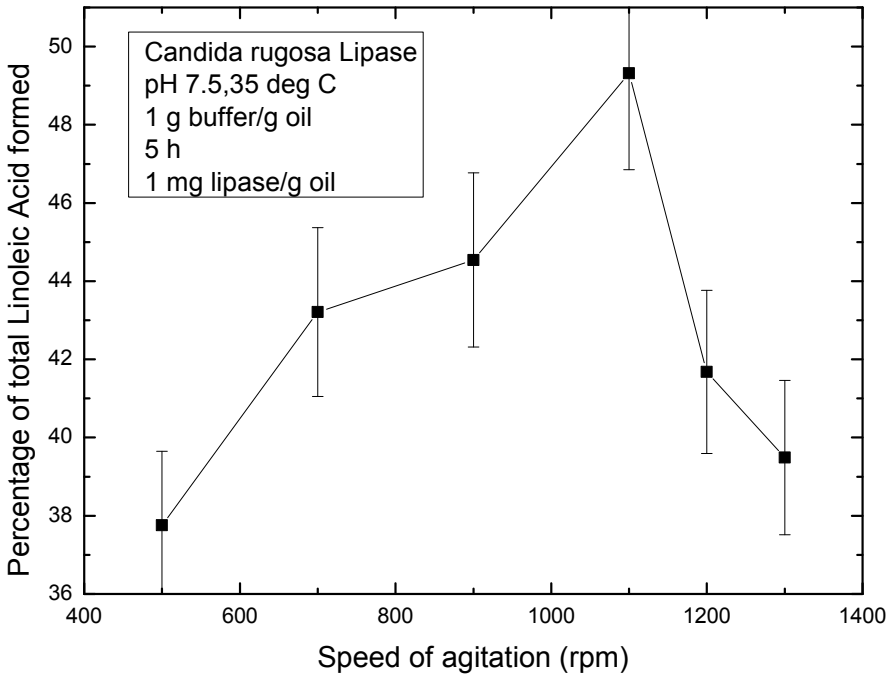


Fig. 4.2 Effect of Agitation speed on 'percentage of total linoleic acid formed' (TSO)

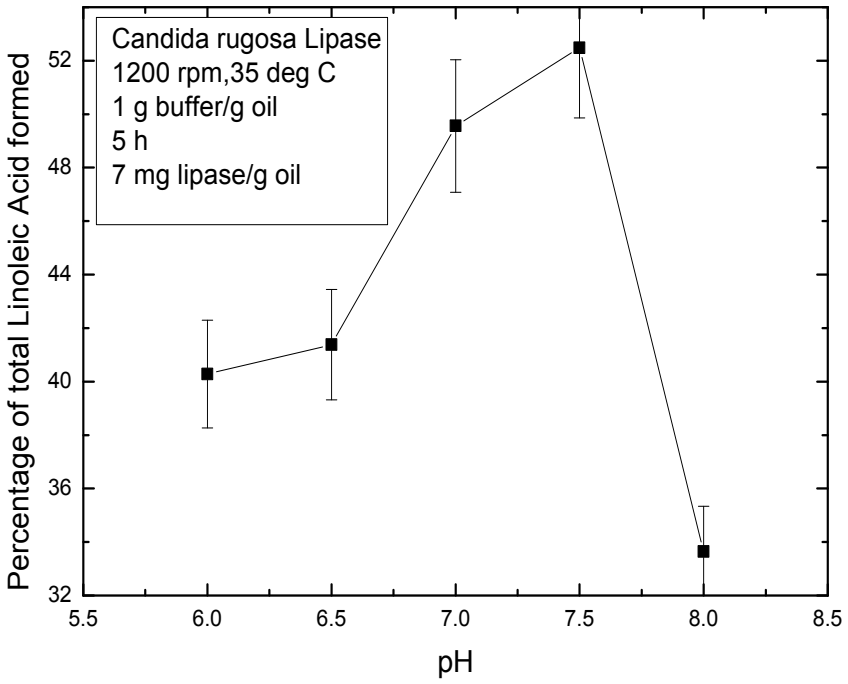


Fig. 4.3 Effect of pH on 'percentage of total linoleic acid formed' (PSO)

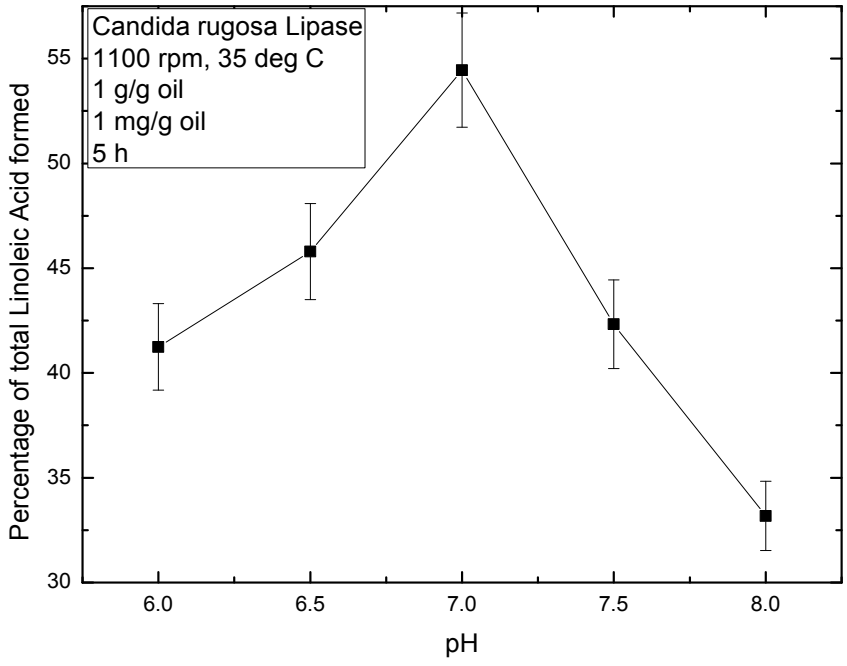


Fig. 4.4 Effect of pH on 'percentage of total linoleic acid formed' (TSO)

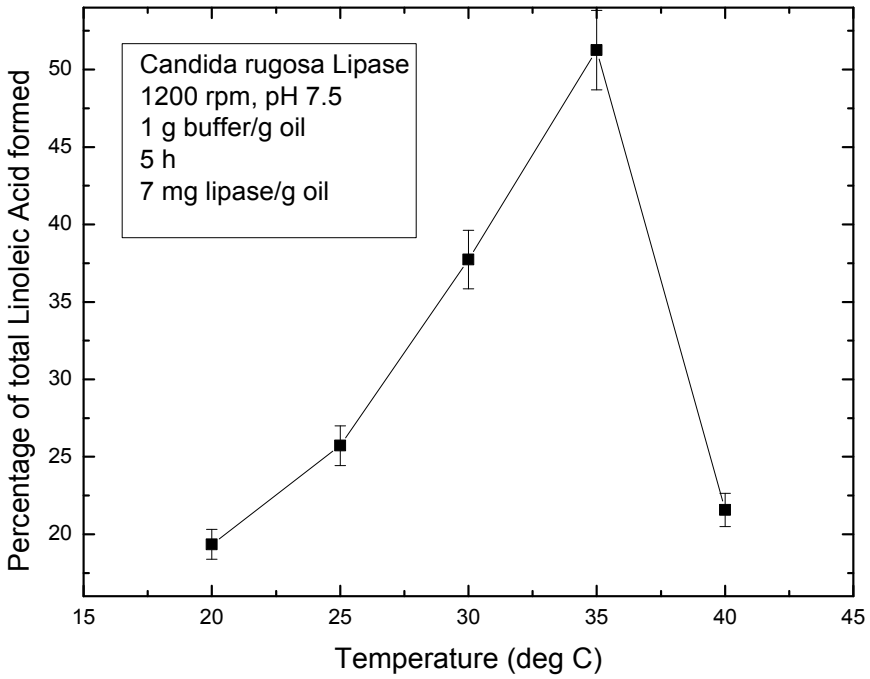


Fig. 4.5 Effect of temperature on 'percentage of total linoleic acid formed' (PSO)

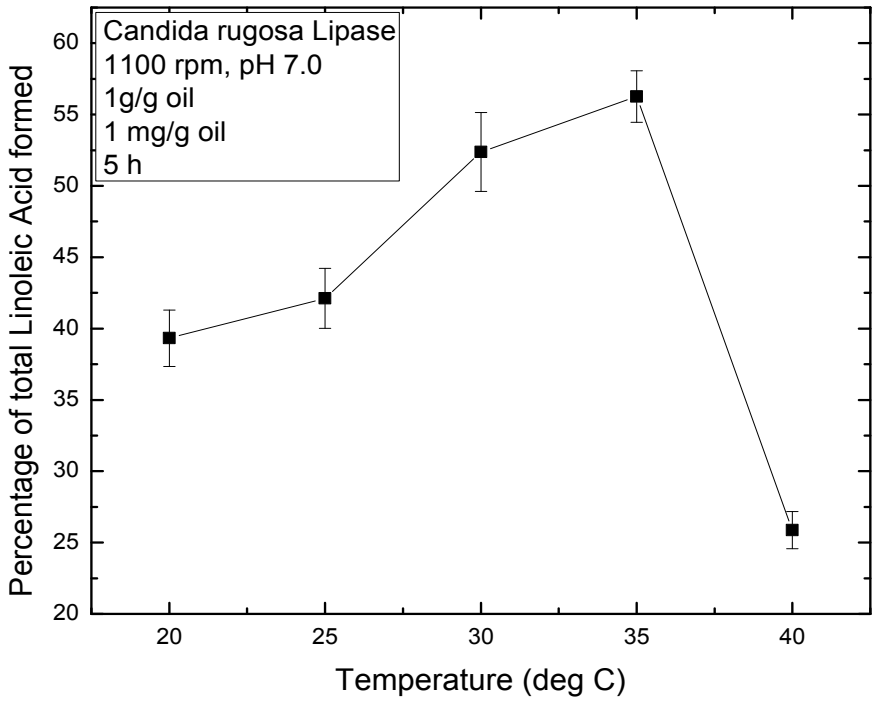


Fig. 4.6 Effect of temperature on 'percentage of total linoleic acid formed' (TSO)

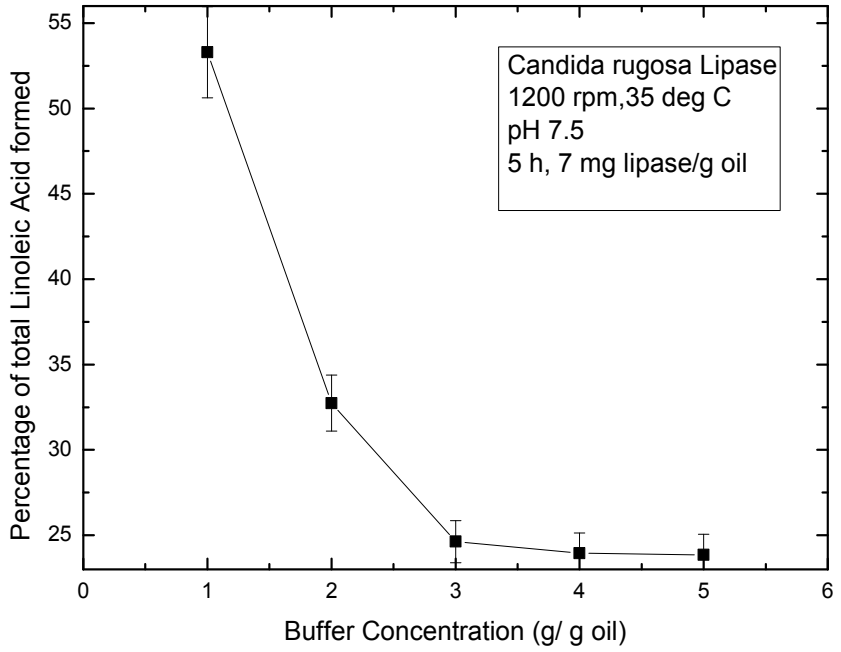


Fig. 4.7 Effect of Buffer Concentration on 'percentage of total linoleic acid formed' (PSO)

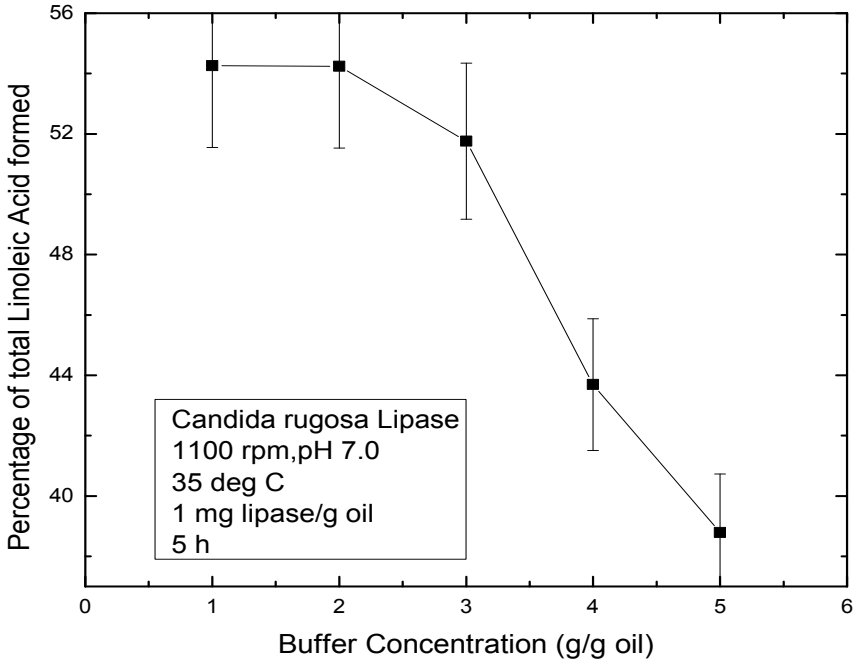


Fig. 4.8 Effect of Buffer Concentration on 'percentage of total linoleic acid formed' (TSO)

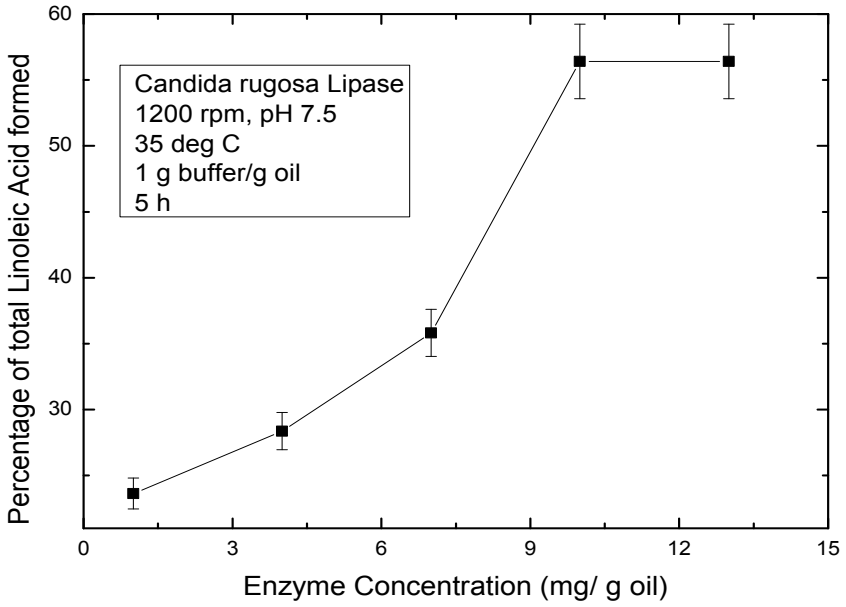


Fig. 4.9 Effect of Enzyme Concentration on 'percentage of total linoleic acid formed' (PSO)

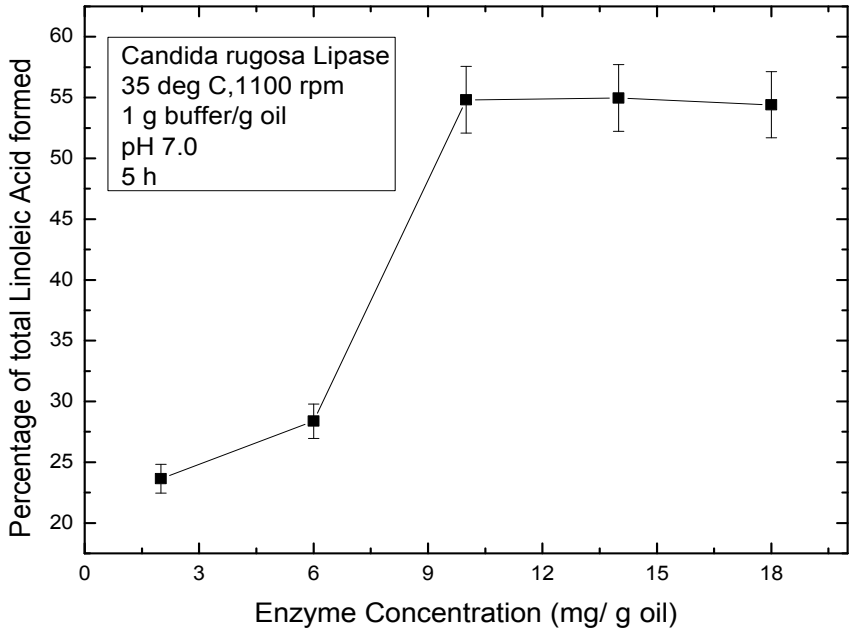


Fig. 4.10 Effect of Enzyme Concentration on 'percentage of total linoleic acid formed' (TSO)

Chapter 5

Experimental Design & Optimization of effective parameters using Response Surface Methodology (RSM)

Chapter 5

Experimental Design & Optimization of effective parameters using Response Surface Methodology (RSM)

Abstract

This chapter describes a detailed optimization study for the production of linoleic acid (%) upon hydrolysis of Pumpkin seed oil. Response Surface Methodology (RSM) with Central Composite Rotatable Design (CCRD) and Desirability Function has been used as a Statistical Design of Experiment (DoE) tool to optimize the production of linoleic acid by simultaneously varying different parameters.

5.1 Introduction

The hydrolysis reaction and parametric studies on hydrolysis of pumpkin and tobacco seed oil has been reported in Chapter 4. The foremost objective of this research work is the evolving and estimation of the RSM approach to better interpret the relationships between the parameters of a lipase catalysed hydrolysis reaction. In the conventional one-dimensional optimization method, which has been implemented most regularly in the literature, variables are varied one at a time while other variables are kept constant. The optimization of experimental conditions using experimental design is broadly functional in a large area of chemical processes. RSM concerted with a five-level-four-factor CCRD is an advantageous statistical technique for exploring complex processes, especially for the optimum yield of product in lipase-catalysed hydrolysis reaction. In this way, the process can be optimized before the scale-up procedure in order to save experimental work, money and time, allowing economically important linoleic acid to be procured at lower cost. Thus, the chief benefit of RSM is the less number of experimental runs required to provide adequate information for statistically satisfactory results [Floros and Chinnan, 1987; Yahiaoui et al. 2011].

5.2 Multivariate Experimental Design

In this study, response surface methodology (RSM) was used for predictive model building and optimization [Montgomery, 2001] for the lipase catalysed hydrolysis reaction. For the optimization study, all experiments were carried out according to statistical designs in order to develop the predictive regression models used for optimization. A 2^4 full factorial central composite rotatable design (CCRD) was employed. A modified quadratic regression

model was derived with satisfactory prediction. Four operating factors were selected as independent variables, namely: Temperature (A) (20-40 ºC), pH (B) (6-8), buffer concentration (C) (1-5 g/g oil) and enzyme concentration (D) (1-13 mg/g oil). Other parameters such as a time and speed of agitation were fixed at 5 h and 1200 rpm respectively. On the other hand, the yield of linoleic acid as dependent output response which is expressed in percent:

$$\text{Percentage of total fatty acid (\%)} = \frac{\text{Net amount of fatty acid in product (g)} \times 100}{\text{Net amount of fatty acid in oil (g)}}$$

where, pumpkin seed oil contains 62.8% [Web ref. 6] linoleic acid and tobacco seed oil contains 76 % [Stanisavljević et al., 2007] linoleic acid.

5.3 Development of regression model equation

'Design Expert' software (Version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) generated the experimental design matrix using the range and levels used in Table 5.1. A total of 30 experimental runs were suggested by the design. This study assimilated four independent variables with five levels and six replicates with run no. 2, 7, 16, 18, 21 and 25 at the centre points. The percentage of total linoleic acid was considered as the response of the design experiments. Experiments were carried out following the design and the responses listed in Table 5.3.

5.4 Model selection and fitting

The models were nominated on the basis of favorable statistical parameters as analyzed by Design Expert software. ANOVA calculates "F-value" and "p-value" to display the significance of model, model terms and lack of fit and on the basis of that modification and model reduction can be done to develop best fitted model for the response (Table No. 5.2).

The experimental data were analyzed by the response surface regression (RSREG) procedure to fit the polynomial equation. Quadratic model of the form shown in Eq. 5.1 was found to be best-fitted as suggested by best lack of fit test, favorable F value, 'prob>F' value, standard deviation and R^2 value.

$$Y = \beta o + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_i Xi^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i \ X_j \qquad (5.1)$$

$$Y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + r \quad (5.2)$$

where, Y was the process response. The coefficient β_0 was the free or offset term intercept. The coefficients β_1 to β_4 signified first order effects for parameters. The coefficients β_{11} to β_{44} symbolized quadratic effects for the variables. The coefficients β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} signified first order interaction effects for the variables. The term 'r' (residual) showed uncertainties or inconsistencies between predicted value and measure value of response [Montgomery, 2001]. The independent or test variables were coded using the actual value by the following equation:

$$x_i = \frac{X_i - X_o}{\partial X} \quad (5.3)$$

where, x_i is dimensionless value of independent variable, X_i represents the real value of independent variables, X_0 is value of the X_i at the centre point and ∂X represents the step change [Box et al., 1978 and Goupy, 1999].

5.5 Results and Discussion

The statistical combination of variables which were used for optimization along with response i.e., 'percentage of total linoleic acid formed' is given in Table 5.2. Also, the summary of ANOVA signifying the results of the quadratic response surface model fitting is displayed in Table 5.3. A very low probability (p-value) of < 0.0001 and the F-value of 49.37 was large enough to validate a very high degree of appropriateness of the model and also direct that the treatment combinations are highly significant [Khuri and Cornell, 1987]. The Model F-value of 49.37 implies the model is significant and there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. A non-significant lack of fit is considered as good. Here, the "Lack of Fit F-value" of 3.00 implies the Lack of Fit is not significant relative to the pure error. There is an 11.77% chance that a "Lack of Fit F-value" this large could occur due to noise. The coefficient of determination (R^2), a measure of goodness of fit of the model, was highly significant at the level of 97.57% (Table 5.3), Also, the "Pred R-Squared" of 0.8937 is in reasonable agreement with the "Adj R-Squared" of 0.9559. This specifies that the model obtained will be able to give a good estimate of the response of the system in the experimental range studied. At the same time a moderately lower value of coefficient of variation (CV = 5.21%) indicated a better precision and

reliability of experiments [Box and Wilson, 1951]. Adequate precision was also found to be 29.963. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is considered to be desirable. A ratio of 29.963 indicates an adequate signal. So, the model can be used to navigate the design space.

RSM leads to the following regression equation, an experimental correlation between 'percentage of total linoleic acid' formed and the process variables in the coded units:

Percentage of total Linoleic Acid formed = $47.42 + 7.34A + 1.06B - 4.04C + 7.61D + 2.48AB - 1.49AD + 1.25BC + 2.62BD + 0.79CD - 0.88A^2 - 0.86B^2 - 2.07C^2 + 1.97D^2$

5.5.1 Comparison of main effects of variables on Response i.e., 'percentage of total linoleic acid formed'

The main (first order) effects of variables on 'percentage of total linoleic acid formed' and the significance of each coefficient as determined by their respective p-values have been provided in Table 5.4. The value of each coefficient suggests the extent of effect it has on each response, whereas its sign indicates whether it has a positive or negative influence on each response. In case of 'percentage of total linoleic acid formed', all main effects are equally significant as their p value is less than 0.01 except pH. Also, all main effect coefficients of the model for 'percentage of total linoleic acid formed' have positive effect except buffer concentration. It shows that first order main effect ($p = < 0.0001$) of temperature is more significant than that of its quadratic main effect ($p = 0.0730$). Both first order effect (0.0464) and quadratic main effect (0.0795) of pH are insignificant. This suggests that an alteration in pH does not significantly affect the response in this case. First order effect ($p = < 0.0001$) of buffer concentration is highly significant while its quadratic main effect is somewhat equally significant ($p= 0.0003$). Similar is the case with enzyme concentration, both first order main effect ($p = < 0.0001$) and quadratic main effect ($p = 0.0005$) are significant. Form this, it can be concluded that buffer and enzyme concentration have a pronounced influence on the response. Predominantly, a minor change in enzyme concentration can lead to a huge variation in response.

Fig 5.1(a) shows the normal (%) probability plot of the residuals. It clearly shows a normal distribution of errors and that these errors are inconsequential. Fig. 5.1(b) shows the predicted values versus actual values for 'percentage of total linoleic acid formed'. As can be understood, the predicted values attained were quite adjacent to the experimental values. The

above discussion evidently exhibits that the regression model developed was successfully in accordance with the experimental data. Besides, the model was also verified for any transformation that could have been applied, but Box-Cox plot did not recommend any transformation for the response.

5.5.2 Comparison of Response 3D Plots and Contour Plots for 'percentage of total linoleic acid formed'

An apparent explanation of the effects of variable interaction on 'percentage of total linoleic acid' can be perceived by observing the response 3D plots and contour plots (Fig. 5.2 –5.6). 3D surface plots shows the degree of deviation of the responses with single and combined effects of two factors (keeping other two factors at zero level) while 2D contour identifies the main interactions that take place between the process parameters from the circular or elliptical shapes of the contours. 2D contour plots are nothing else but the representation of 3D plots. Surface restrained by the smallest ellipse points to maximum response.

Fig.5.2 and Fig.5.3 shows the simultaneous interactive effect of temperature with pH and enzyme concentration on 'percentage of total linoleic acid formed'. It shows a strong interaction of temperature with pH as well as with enzyme concentration. From these two figures it can be depicted that an optimum range of temperature is 31 to 35ºC. Fig. 5.4 and 5.5 shows an interaction of pH with buffer and enzyme concentration respectively. There is a strong interaction between pH and enzyme concentration as is also supported by it p-value i.e., 0.0005. From these two figures it is clear that an optimum range of pH is 6.8 to 7.0. Fig. 5.6 displays an interaction plot between enzyme and buffer concentration. From this figure it is clearly indicated that there is no significance interaction between these two terms. There is also a presence of saddle point. From this figure optimum range of enzyme and buffer concentration can be depicted to be 9.0 to 10.0 mg/g oil and 2.0 to 2.3 g/g oil respectively.

5.5.3 Numerical optimization of 'percentage of total linoleic acid formed'

Our key objective is to maximize production of linoleic acid with minimal use of enzyme and at minimum temperature. And in this way a cost effective process can be procured. Preeminent solutions with maximum desirability were tabularized for individual set of optimized variables and response in Table 5.5.

The optimization by means of desirability function suggests some criteria. Primarily,

a higher value of distinct desirability function (d_i) means that the nearby the predicted responses are to our target requirements, the closer the specific desirability will be to a value of 1. Secondly, a greater value for overall desirability function (D) means that the composite desirability associates the individual desirability into an overall value, and reflects the relative significance of the responses. The greater the desirability the closer it will be to a value of 1. Finally, it is the establishment of optimal values for process parameters. Desirability for each factors along with generated responses are shown in a typical desirability plot (Figure 5.7) for a particular optimization solution with their corresponding desirability values. It shows desirability for 'percentage of total linoleic acid formed' as 0.829 and combined desirability (D) as 0.939 which displays a high degree of attainment in terms of anticipated goal. The most desirable reaction condition for maximum production of 'percentage of total linoleic acid' (93.9%) is temperature = 35°C, pH = 7.5, buffer concentration = 4 g/g oil and enzyme concentration = 10 mg/g oil. Under this condition, a moderate temperature condition is being used to obtain 82.9% response while experimentally under similar conditions only 63.53% of response was noted, which indicates that it is a cost-effective process.

5.6 Conclusion

The enzymatic hydrolysis of non-conventional oil resources are of great importance for oleochemical industries leading to the production of useful fatty acids. The hydrolysis reaction was investigated in details under various conditions of varying parameters. Response Surface Methodology (RSM) was used for predictive model building and optimization. Optimization led by single variable method showed an optimum temperature of 35°C and RSM also predicted it to be in the range of 31 to 35°C. This implies that RSM recommends a moderate temperature for maximization of linoleic acid production. Similarly, optimum pH was noticed at 7.5 by single optimization method which is also supported by RSM as its optimum range is 6.8 to 7.0. RSM finds optimum buffer concentration in the range of 2.0 to 2.3 g/g oil but according to single variable optimization method an optimum buffer concentration was examined at 1.0 g/g oil i.e., RSM projected optimum buffer concentration is on larger side. Conferring to optimum enzyme concentration of 10.0 mg/g oil by single variable optimization, RSM also approached optimum enzyme concentration from 9.0 to 10.0 mg/g oil showing no significant change.

Numerical optimization computed the overall optimization and resulted to 0.939 of

combined desirability and 82.9% response under optimum reaction conditions indicating a cost effective process.

From these optimum values of different parameters it can be conferred that RSM is an advantageous method as cumulative effects of different parameters shows a maximum response. Hence, the use of RSM with CCRD and desirability function facilitated in reaching the overall optimal solutions and in safely analyzing the interactive effects of selected parameters on ‘percentage of total linoleic acid formed’ for improved estimation of the process.

Table No. 5.1 Experimental ranges and levels of the Independent Variables

Independent Variables	Factors	Range and Levels				
		-2	**-1**	**0**	**1**	**2**
Temperature (°C)	A	20	25	30	35	40
pH	B	6	6.5	7	7.5	8
Buffer Conc.(g/g oil)	C	1	2	3	4	5
Enzyme Conc. (mg/g oil)	D	1	4	7	10	13

Table No. 5.2 Experimental Design Matrix for Central Composite Design with four variables in Actual Units along with the Observed Response (Percentage of total linoleic acid formed)

Std	Run	Temp. (°C)	pH	Buffer Conc. (g/g oil)	Enzyme Conc. (mg/ g oil)	Total Linoleic Acid formed (%)
26	1	25	6.5	2	4	41.82
10	2	35	6.5	2	4	49.14
12	3	25	7.5	2	4	28.02
17	4	35	7.5	2	4	49.64
9	5	25	6.5	2	10	48.23
30	6	35	6.5	2	10	56.01
13	7	25	7.5	2	10	50.18
24	8	35	7.5	2	10	66.89
6	9	25	6.5	4	4	24.43
29	10	35	6.5	4	4	43.05
28	11	25	7.5	4	4	22.62
15	12	35	7.5	4	4	46.68
1	13	25	6.5	4	10	43.02
8	14	35	6.5	4	10	49.19
19	15	25	7.5	4	10	46.34
27	16	35	7.5	4	10	63.53
14	17	20	7	3	7	29.78
5	18	40	7	3	7	58.17
23	19	30	6	3	7	42.48
4	20	30	8	3	7	45.64
20	21	30	7	3	1	39.21
11	22	30	7	3	13	71.56
22	23	30	7	1	7	50.68
3	24	30	7	5	7	27.72
21	25	30	7	3	7	44.63
16	26	30	7	3	7	46.58
2	27	30	7	3	7	48.49
18	28	30	7	3	7	47.86
7	29	30	7	3	7	48.28
25	30	30	7	3	7	48.65

Table No. 5.3 Analysis of Variance (ANOVA) for the Quadratic Model

Sources of variations	Sum of squares	Degrees of freedom	Mean square	F-value	Probability
Model	3681.85	13	283.22	49.37	< 0.0001
Residual	91.79	16	5.74		
Error	12.08	5	2.42		
COR. Total	3773.64	29			

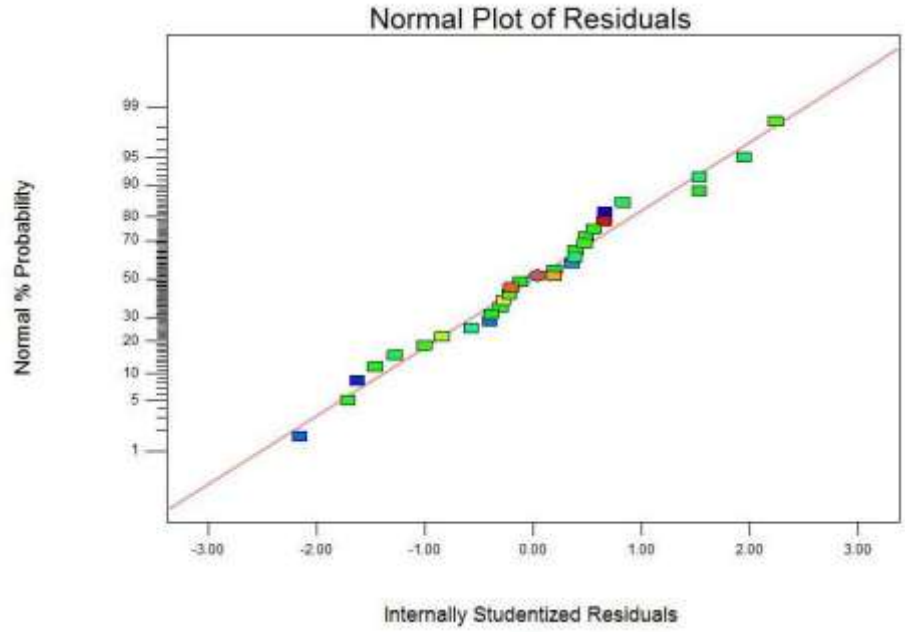
Standard Deviation = 2.4; CV % = 5.21; R^2 = 0.9757; Adj R^2 = 0.9559, Pred R^2 = 0.8937

Table No. 5.4 The Least Squares Fit and Significance of Regression Coefficients in Full Factorial Central Composite Design

Model term	Regression Coefficient	Standard Error	p-value
Intercept	47.42	0.98	-
A-Temperature	7.34	0.49	< 0.0001
B-pH	1.06	0.49	0.0464
C-Buffer Concentration	-4.04	0.49	< 0.0001
D-Enzyme Concentration	7.61	0.49	< 0.0001
AB	2.48	0.6	0.0008
AD	-1.49	0.6	0.0246
BC	1.25	0.6	0.0537
BD	2.62	0.6	0.0005
CD	0.79	0.6	0.2067
A^2	-0.88	0.46	0.0730
B^2	-0.86	0.46	0.0795
C^2	-2.07	0.46	0.0003
D^2	1.97	0.46	0.0005

Table 5.5: Optimized reaction conditions based on selected criteria

Criteria	Temperature (°C)	pH	Buffer Conc. (g/ g oil)	Enzyme Conc. (mg/ g oil)	Response	Desirability
'Percentage of linoleic acid formed' is maximum, pH maximum and buffer concentration maximum	35	7.5	4	10	82.9	0.94
'Percentage of linoleic acid formed' is maximum and buffer concentration maximum	35	7.5	4	10	63.2	0.911
'Percentage of linoleic acid formed' is maximum, pH maximum and enzyme concentration minimum	35	7.5	2.13	4	51.3	0.837
'Percentage of linoleic acid formed' is maximum, pH maximum, buffer conc. maximum and enzyme minimum	35	7.5	4	4	44.1	0.814



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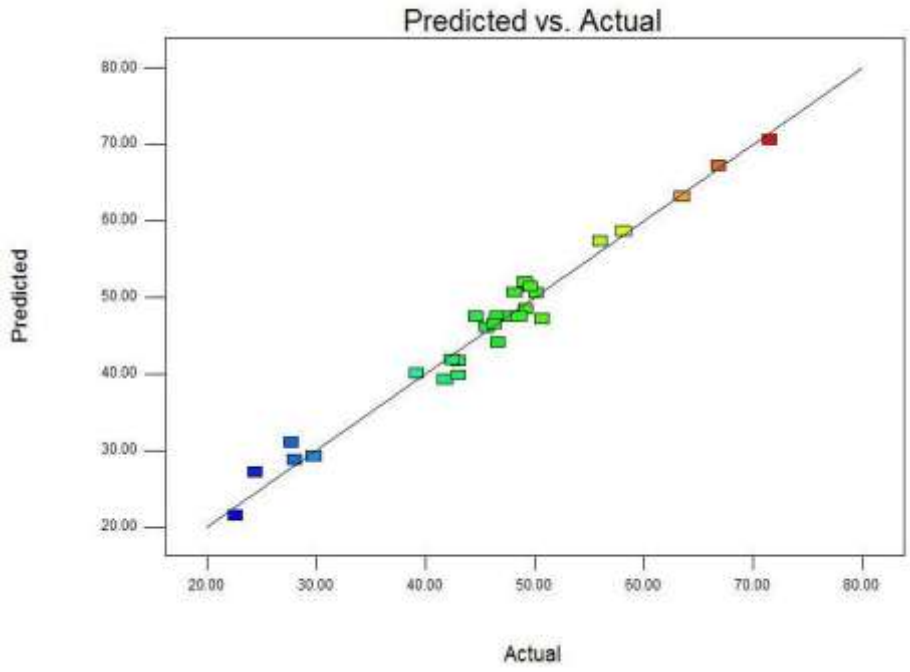
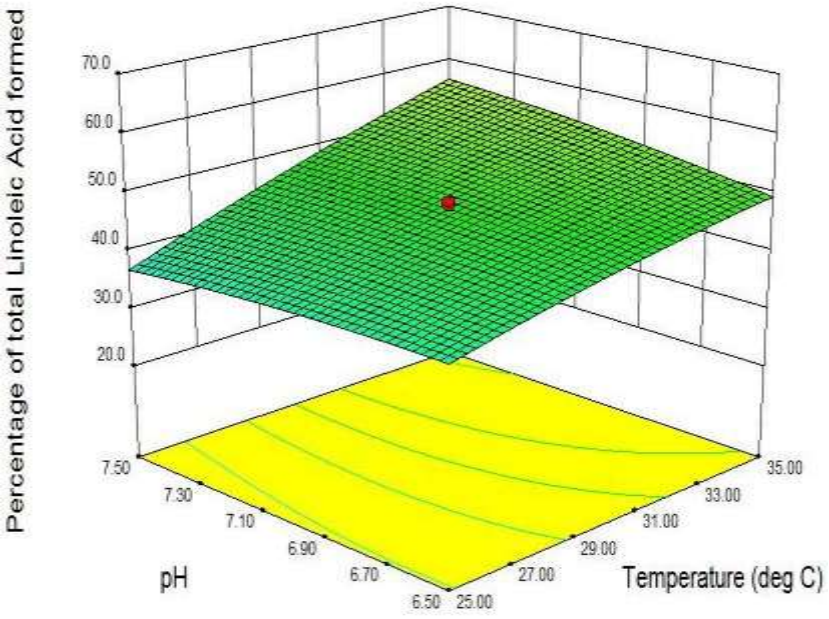


(b)

Fig No. 5.1 Model validation plot (a) Normal % probability plot of internally studentized residuals for 'percentage of total linoleic acid formed (b) Relationship between the observed and the predicted 'percentage of total linoleic acid formed



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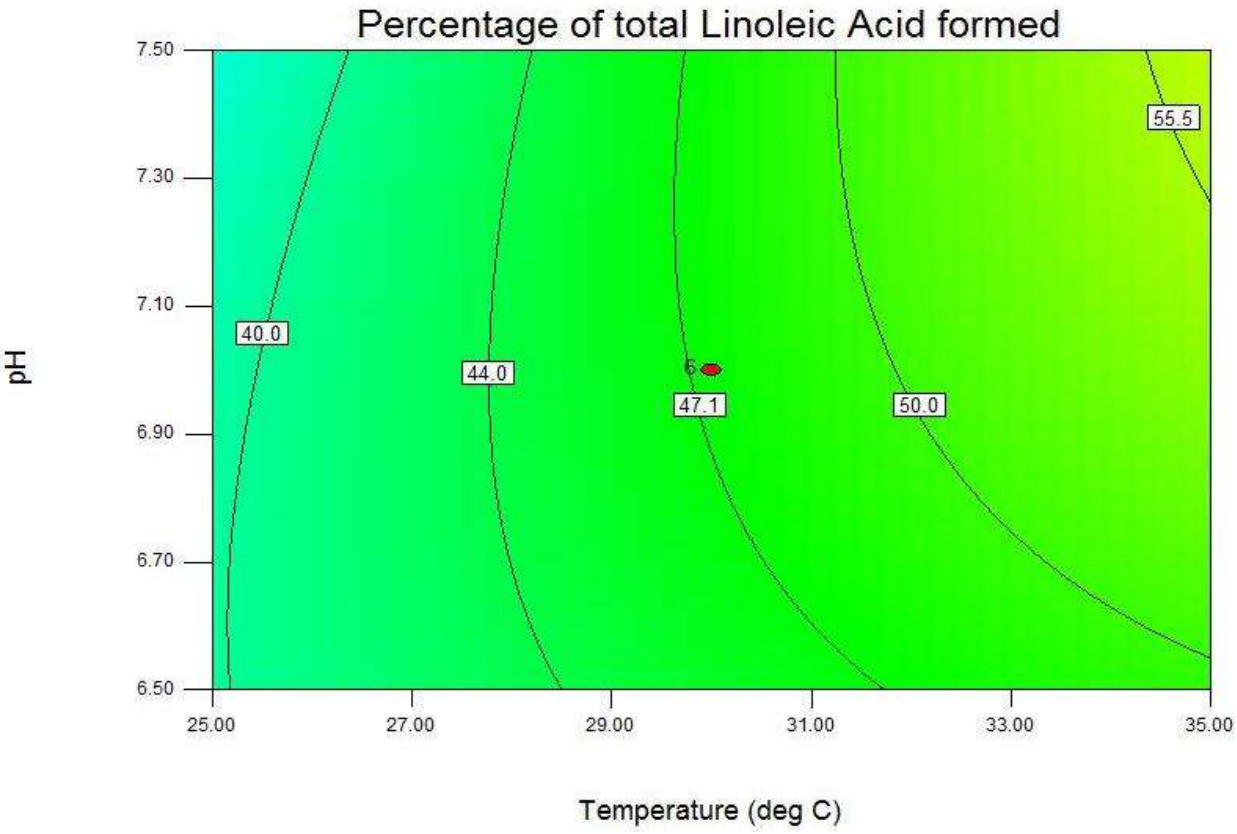
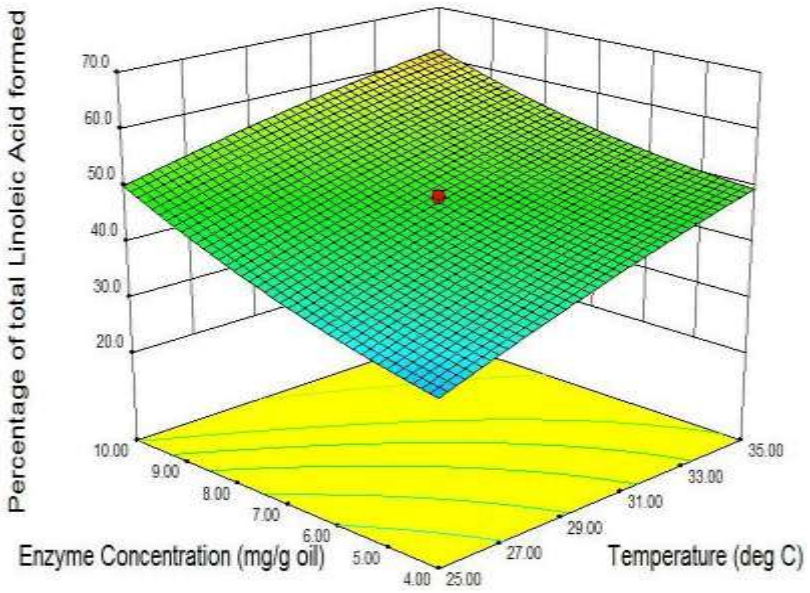


(b)

Fig.No. 5.2 Simultaneous interactive effect of temperature and pH on 'percentage of total linoleic acid formed'



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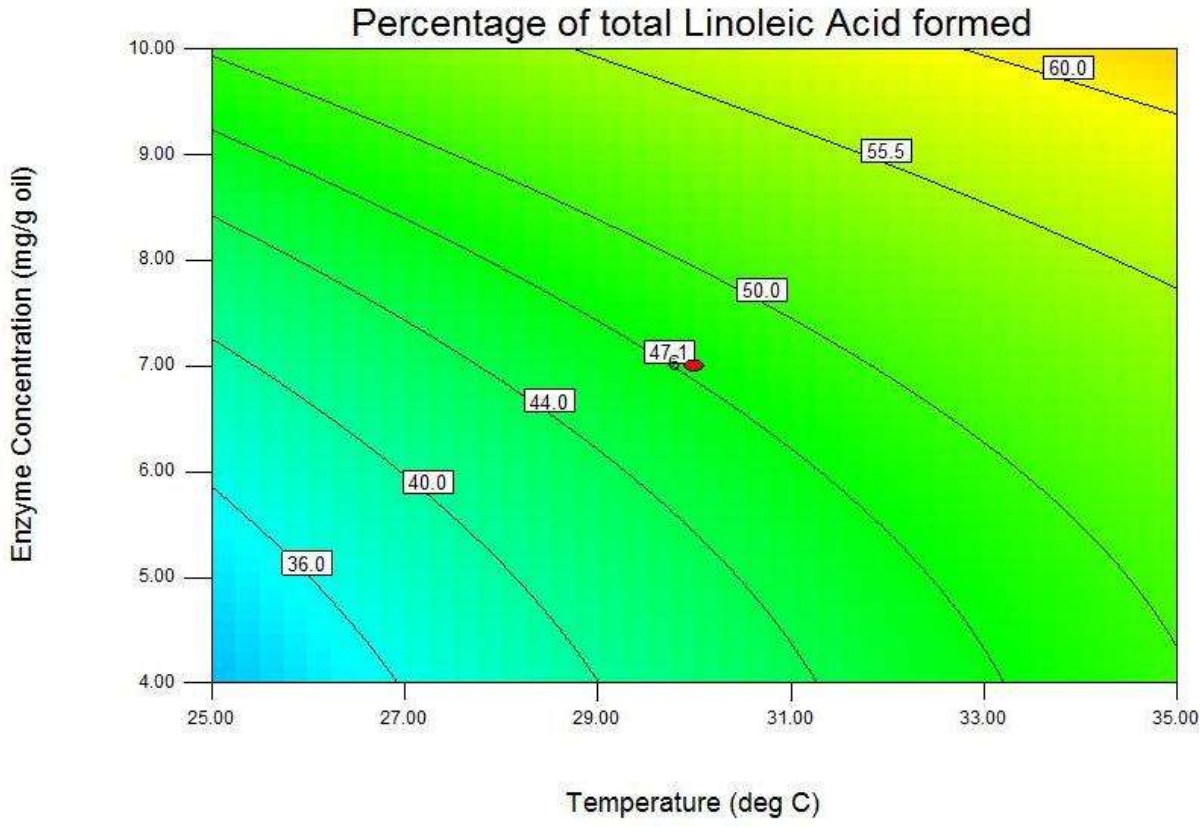
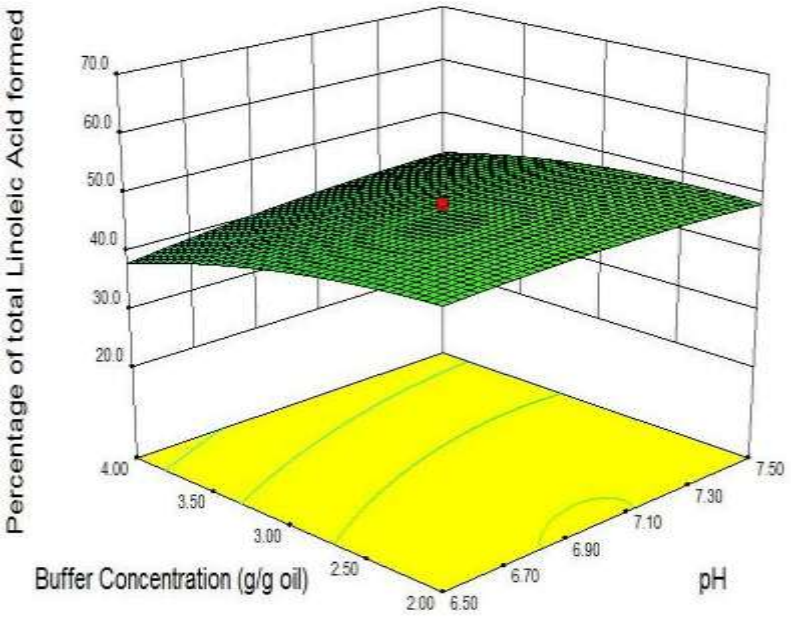


(b)

Fig.No. 5.3 Simultaneous interactive effect of temperature and enzyme concentration on 'percentage of total linoleic acid formed'



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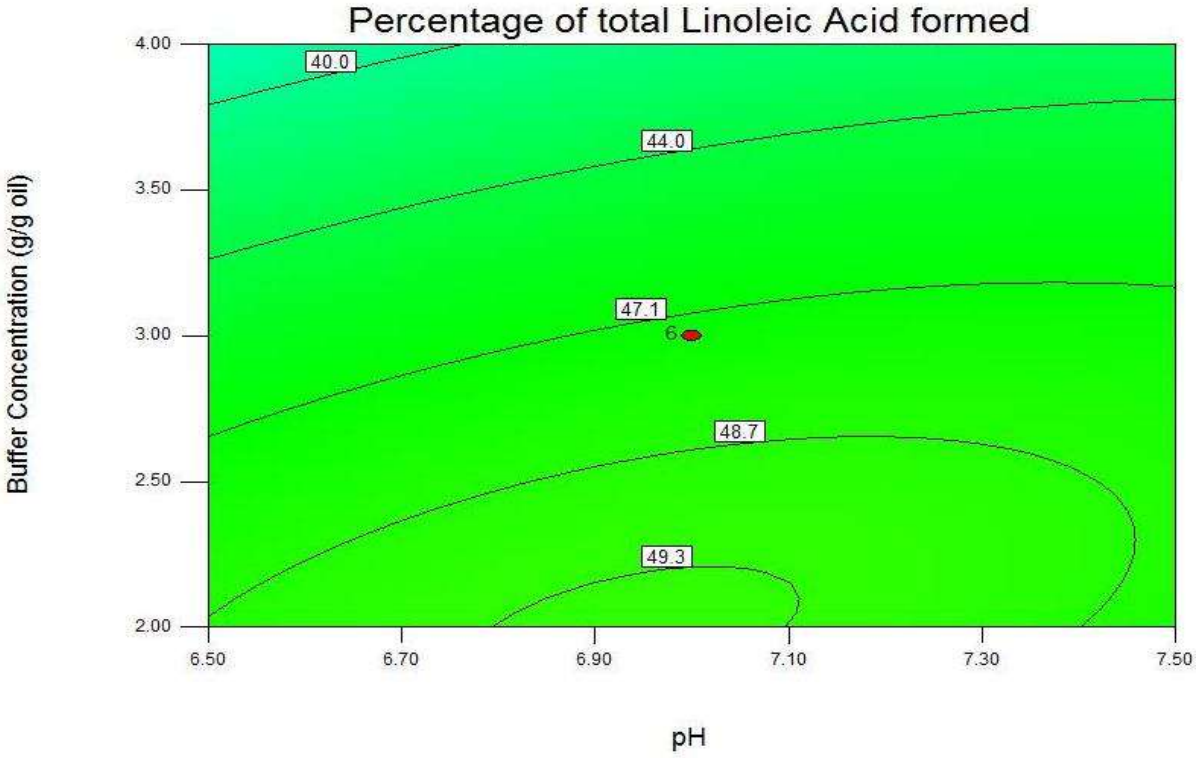
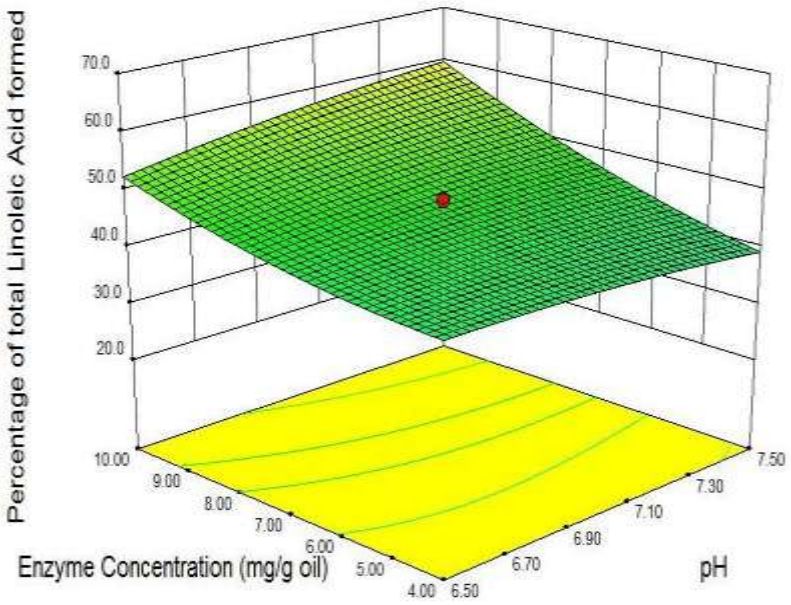


(b)

Fig.No. 5.4 Simultaneous interactive effect of pH and buffer concentration on 'percentage of total linoleic acid formed'



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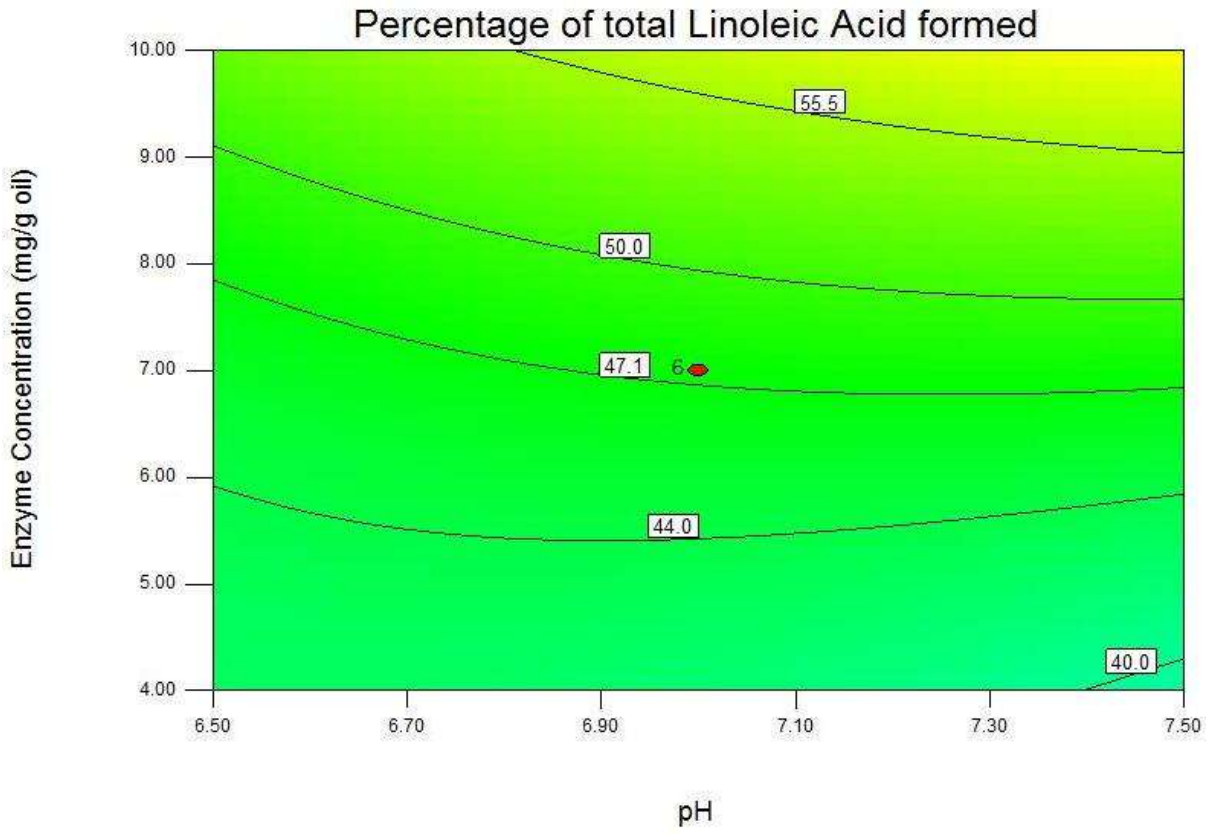
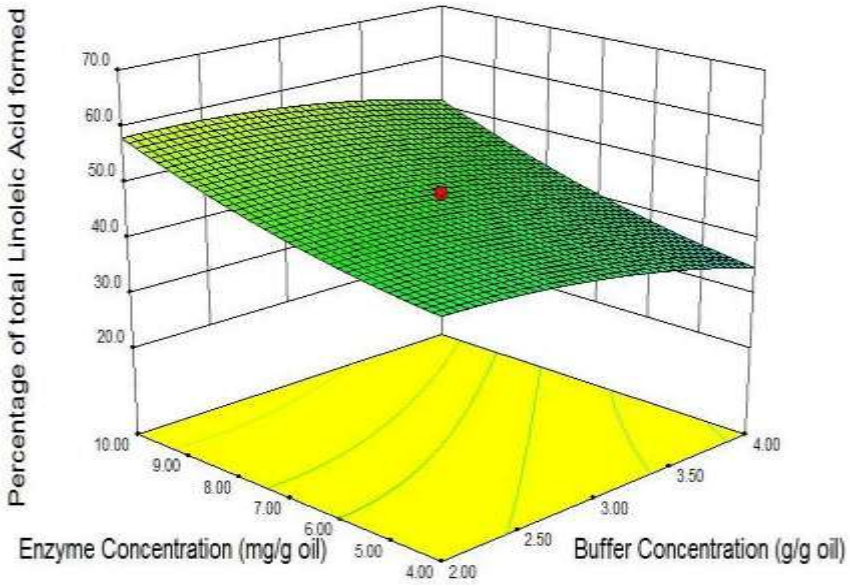


(b)

Fig.No. 5.5 Simultaneous interactive effect of pH and enzyme concentration on 'percentage of total linoleic acid formed'



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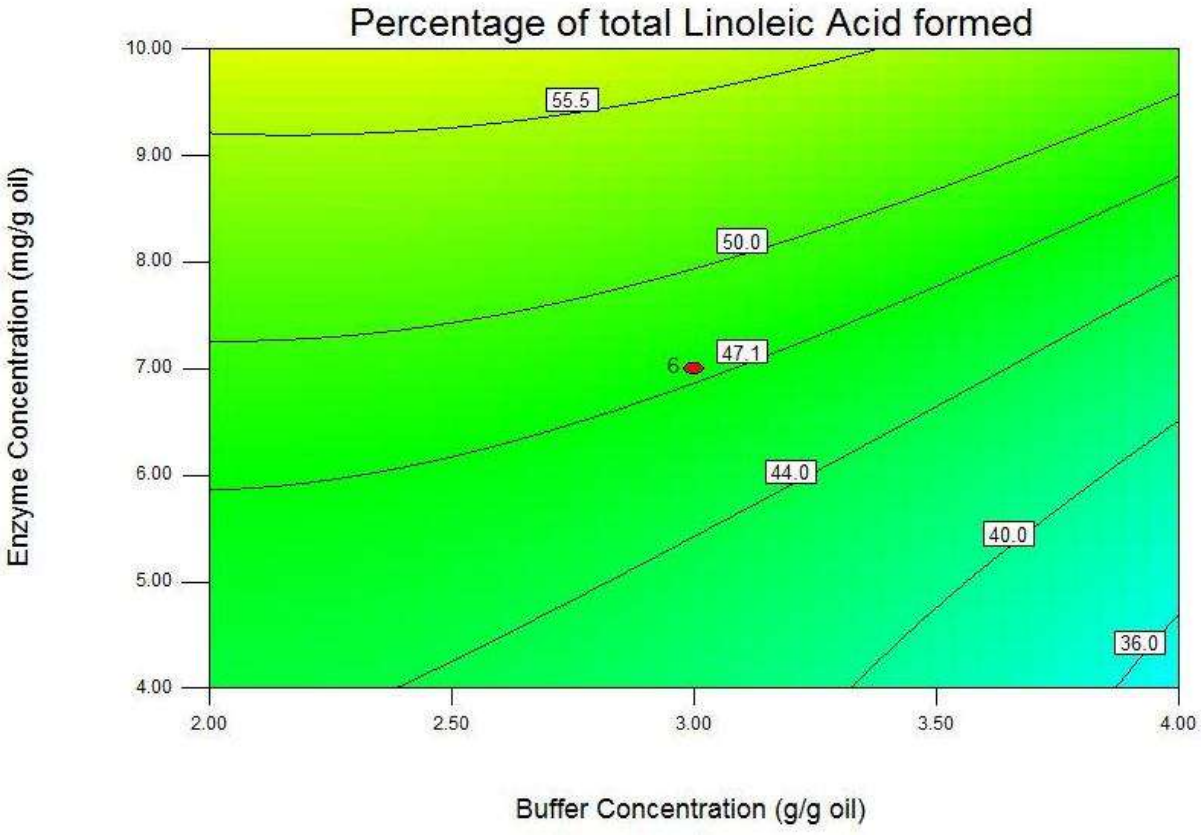


(b)

Fig.No. 5.6 Simultaneous interactive effect of buffer and enzyme concentration on 'percentage of total linoleic acid formed'

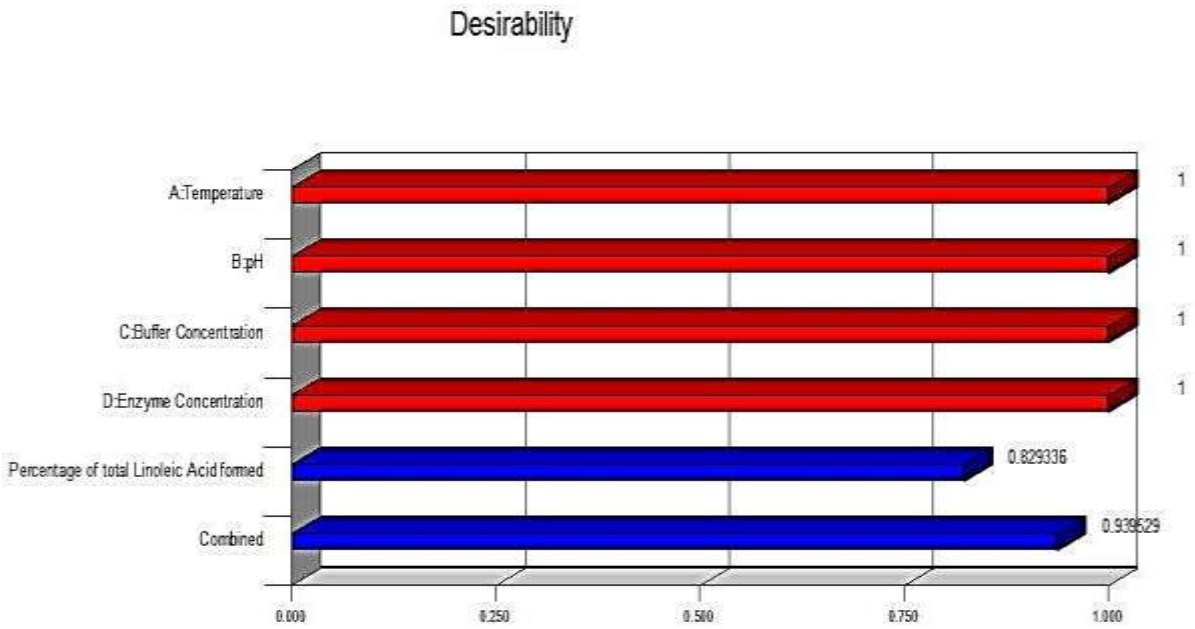


Fig. 5.7 Desirability plot for a typical optimization solution

Chapter 6

Optimization Study of Enzymatic Production of Linoleic acid by Hydrolysis of Tobacco seed oil

Chapter 6

Optimization Study of Enzymatic Production of Linoleic acid by Hydrolysis of Tobacco seed oil

Abstract

The overall purpose of this study is to model and optimize the production of linoleic acid by hydrolysis of tobacco seed oil. Response surface methodology (RSM) was used to estimate the effect of the most significant factors, such as temperature, pH, buffer concentration and enzyme concentration on the hydrolysis process of tobacco seed oil for maximization of the production of linoleic acid. Response Surface Methodology (RSM) with Central Composite Rotatable Design (CCRD) and Desirability Function has been used as a Statistical Design of Experiment (DoE) tool to optimize the 'percentage production of linoleic acid'.

6.1 Introduction

In this chapter Response surface methodology (RSM) was used for analytical model building and optimization [Montgomery, 2001] for the production of linoleic acid. For the optimization study, all experiments were performed agreeing to statistical designs in order to develop the predictive regression models used for optimization. A 2^4 full factorial central composite rotatable design (CCRD) was applied. A modified quadratic regression model was derived with satisfactory assessment. The objective of this study is to well understand the interactions between the factors affecting linoleic acid production, and to control the optimum environments for the maximum production of linoleic acid. The main advantage of using RSM for optimization is to reduce the number of experimental runs necessary to deliver appropriate information for statistically suitable results.

6.2 Modeling and Optimization

A reaction, such as the hydrolysis of oil, which is concurrently influenced by more than one factor, can be incompletely understood with OVAT approach [Yuan et al., 2008]. So, the development of a mathematical model with optimization study for such kind of important complex reaction is highly desired for the betterment of the process.

Alternately, Design of Experiments (DOE) can be approached for optimization study (Chapter 5). Response Surface Methodology (RSM) with Central Composite Rotatable

Design (CCRD) was effectively applied to validate the various interactions of responsible factors for the production of total linoleic acid. Besides, the desirability function for optimization of the complete process was employed in order to develop an effective method for attaining maximum production of linoleic acid by combination of all optimized input factors.

6.3 Multivariate experimental design

Temperature, pH, buffer concentration and enzyme concentration was selected as variables. Four operating factors were selected as independent variables, namely: Temperature (A) (20-40 ºC), pH (B) (6-8), enzyme concentration (C) (2-14 mg/g oil) and buffer concentration (D) (1-5 g/g oil). Effect of agitation speed was shown to not affect the process so it was kept constant at 1100 rpm. Agitation speed was not used as a parameter for design of process. Also, all responses were calculated for 5 h of reaction time. 'Percentage of total linoleic acid formed' was taken as single response for the present experimental design.

A typical RSM with five level four-factor full factorial CCRD was applied in this work. Table 6.1 lists the range and levels of the four independent variables studied.

6.4 Development of regression model equation

Design Expert software (version 8.0.7.1) originated the experimental design matrix using the range and levels used in Table 6.1. A total of 30 experimental runs were suggested by the design. Runs 10, 20, 7, 16, 2 and 29 are at the centre point of the design. Experiments were preceded by the following design and the responses which are listed in Table 6.2.

6.5 Model selection and fitting

The models were approved on the basis of positive statistical parameters as analysed by Design Expert software. ANOVA calculates "F-value" and "p-value" to illustrate the significance of model, model terms and lack of fit and on the basis of that modification and model reduction can be done to acquire best fitted model for the response.

Model fitting for 'percentage of total linoleic acid formed': Quadratic model of the form shown in Eq. 6.1 was found to be best-fitted as submitted by best lack of fit test, favourable F value, "prob> F" value, standard deviation and R^2 value.

$$Y = \beta o + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_i Xi^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i \ X_j \qquad (6.1)$$

$$Y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + r \quad (6.2)$$

where, Y is the response of dependent variable ('percentage of total linoleic acid formed' in this case), β_0 is constant, βi is regression coefficient for linear effects, β_{ii} is quadratic coefficient and β_{ij} is interaction coefficient.

The summary of ANOVA representing the results of the quadratic response surface model fitting is shown in (Table 6.2).

With a very low probability of 0.0001, the F-value of 25.89, signifying that there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. It was large enough to validate a very high degree of suitability of the model and also to direct that the treatment combinations are highly significant. The "Lack of Fit F-value" of 0.66 implies there is a 73.15% chance that a "Lack of Fit F-value" this large could occur due to noise. The coefficient of determination (R^2), a measure of goodness of fit of the model, was very highly significant at the level of 96.03% (Table 6.2), indicating only 3.97% of the total inconsistency was not explicated by the regression in the model. Also predicted R^2 of 0.8451 is in reasonable agreement with adjusted R^2 of 0.9232. This postulates that the model attained will be able to give a strongly good evaluation of the response of the system in the experimental range considered. At the same time a low value of coefficient of variation (CV = 7.44%) showed a high degree of precision and a good agreement of consistency of experimental values [Box and Wilson, 1951; Akhnazarova et al., 1982]. Adequate precision (27.227) was also found to be much greater than 4. So, the model can be used to navigate the design space. Table 6.4 displays the least squares fit and significance of regression coefficients.

Fig. 6.1(a) shows the Normal plot of residuals for response. It was normally distributed and exhibited no aberration of the variance. Predicted versus actual values for 'percentage of total linoleic acid formed' is shown in Fig. 6.1(b) As can be seen, the predicted values attained were pretty close to the experimental values, indicating that the model developed was successful in validating the correlation between the reaction condition variables to response. Besides, the model was also verified for any transformation that could have been applied, but Box-Cox plot did not recommend any transformation for the response.

The final regression model in terms of coded factors has been expressed by the following second order polynomial equation:

Percentage of total Linoleic Acid formed = 49.41 + 7.06A + 0.086B + 3.41C – 6.06D + 1.1AB + 3.28AC – 3.3AD + 3.18BC – 0.32BD – 4.66CD – $5.97A^2$ + $0.51B^2$ + $1.11C^2$ + $2.17D^2$

6.6 Results and Discussions

6.6.1 Comparison of main effects of variables on 'percentage of total linoleic acid formed'

The main (first order) effects of variables on 'percentage of total linoleic acid formed' and the significance of each coefficient as determined by their respective p-values have been provided in Table 6.4. In case of 'percentage of total linoleic acid formed', only main effects of enzyme and buffer concentration are significant as its p value is less than 0.01. Also, all main effect coefficients of the model for 'percentage of total linoleic acid formed' have positive effect except buffer concentration. Both the first order main effect (p = < 0.0001) and quadratic main effect (p = < 0.0001) of temperature are highly significant. Both first order effect (0.9068) and quadratic main effect (0.4602) of pH are insignificant. This suggests that an alteration in pH does not considerably affect the response in the selected range. First order effect (p = 0.0003) of enzyme concentration is highly significant while its quadratic main effect (p= 0.1206) is not significant at all. In case with buffer concentration, first order main effect (p = < 0.0001) is greatly significant and its quadratic main effect (p = 0.0058) is also significant. Form this, it can be concluded that temperature, buffer and enzyme concentration have a prominent effect on the response. Mainly, a slight change in temperature, buffer and enzyme concentration can lead to a large deviation in response.

6.6.2 Comparison of response 3D plots and contour plots for 'percentage of total linoleic acid formed'

A clear interpretation of effects of variables interaction on response can be made through 3D and contour plots which have been shown in Fig. 6.2- 6.7. Fig. 6.2 to 6.4 shows the interaction of temperature with pH, enzyme concentration and buffer concentration respectively with the presence of saddle point in each case. Fig 6.3 and 6.4 clearly indicates a good interaction between two variables. But Fig. 6.2 which shows an interaction between temperature and pH indicates no significance interaction at all which is also supported by its

p-value (0.2389). From Fig. 6.2-6.4 it can be concluded that optimum temperature falls in the range of 31 to 35ºC. Fig. 6.5-6.6 displays an interaction plot of pH with enzyme and buffer concentration indicating a significant interaction between pH and enzyme concentration while a non-significant interaction between pH and buffer concentration with the presence of saddle point and stationary point in respective cases. From these figures an optimum range of pH can be seen in 7.3 to 7.5. Fig 6.7 indicates a very strong interaction of enzyme and buffer concentration with the presence of saddle point. From this figure in can be perceived that an optimum range of enzyme concentration is 9.0 to 11.0 mg/g oil and buffer concentration is 3.0 to 4.0 g/g oil which is also supported by other contour and 3D plots mentioned here.

6.6.3 Numerical optimization of 'percentage of total linoleic acid formed'

Our main objective is to maximize production of linoleic acid with minimal use of enzyme and at minimum temperature. And in this way a cost effective process can be procured. Leading solutions with maximum desirability were organized for individual set of optimized variables and response in Table 6.5.

Desirability for each factors along with generated responses are shown in a typical desirability plot (Fig. 6.8) for a particular optimization solution with their corresponding desirability values. It shows desirability for 'percentage of total linoleic acid formed' as 0.610 and combined desirability (D) as 0.883 which displays a high degree of attainment in terms of anticipated goal. The most desirable reaction condition for maximum production of 'percentage of total linoleic acid' (61.0%) is temperature = 33.62°C (approx. 34°C), pH = 7.5, enzyme concentration = 6.0 g/g oil and buffer concentration = 2.0 mg/g oil. Under this condition, a moderate temperature condition is being used to obtain 61.0% response which indicates that relatively it is a cost-effective process.

6.7 Conclusion

The hydrolysis reaction was explored in details under many conditions of varying parameters. Response Surface Methodology (RSM) was used for analytical model building and optimization. Optimization led by single variable method showed an optimum temperature of 35°C and RSM also predicted it to be in the range of 31 to 35°C. This implies that RSM recommends a moderate temperature for maximization of linoleic acid production from tobacco seed oil. Similarly, optimum pH was noticed at neutral pH, 7.0 by single optimization method while by RSM its optimum range is slightly towards acidic range from 7.3 to 7.5. RSM finds optimum buffer concentration in the range of 3.0 to 4.0 g/g oil but

according to single variable optimization method an optimum buffer concentration was examined at 1 g/g oil i.e., RSM predicted optimum buffer concentration on a greater side. Discussing about optimum enzyme concentration of 14 mg/g oil by single variable optimization, RSM approached optimum enzyme concentration from 9.0 to 11.0 mg/g oil showing a much little amount of enzyme concentration needed for the production of linoleic acid.

Numerical optimization computed the overall optimization and resulted to 0.883 of combined desirability under optimum reaction conditions indicating a cost effective process.

From these optimum values of different factors it can be conferred that RSM is a useful method as collective effects of different parameters displays a maximum response. Hence, the use of RSM with CCRD and desirability function enabled in reaching the overall optimal solutions and in safely investigating the interactive effects of certain parameters on 'percentage of total linoleic acid formed' for superior estimation of the process.

Table No. 6.1 Experimental ranges and levels of the Independent Variables

Independent Variables	Factors	Range and Levels				
		-2	**-1**	**0**	**1**	**2**
Temperature (°C)	A	20	25	30	35	40
pH	B	6	6.5	7	7.5	8
Enzyme Conc.(g/g oil)	C	2	6	10	14	18
Buffer Conc. (mg/g oil)	D	1	2	3	4	5

Table No. 6.2 Experimental Design Matrix for a Central Composite Design with four variables in Actual Units along with the Observed Response (Percentage of total linoleic acid formed)

Std	Run	Temp (°C)	pH	Enzyme Conc. (mg/ g oil)	Buffer Conc. (g/g oil)	Total Linoleic Acid formed (%)
21	1	25	6.5	6	2	44.42
17	2	35	6.5	6	2	49.35
6	3	25	7.5	6	2	34.12
30	4	35	7.5	6	2	48.44
3	5	25	6.5	14	2	44.26
9	6	35	6.5	14	2	72.11
1	7	25	7.5	14	2	48.15
18	8	35	7.5	14	2	78.79
8	9	25	6.5	6	4	45.4
12	10	35	6.5	6	4	47.15
5	11	25	7.5	6	4	35.53
19	12	35	7.5	6	4	39.66
13	13	25	6.5	14	4	33.11
28	14	35	6.5	14	4	41.13
25	15	25	7.5	14	4	37.42
27	16	35	7.5	14	4	48.49
4	17	20	7	10	3	9.85
15	18	40	7	10	3	43.25
22	19	30	6	10	3	50.39
11	20	30	8	10	3	54.59
23	21	30	7	2	3	49.28
26	22	30	7	18	3	60.51
14	23	30	7	10	1	72.58
24	24	30	7	10	5	45.69
10	25	30	7	10	3	56.61
20	26	30	7	10	3	48.54
7	27	30	7	10	3	44.46
16	28	30	7	10	3	49.76
2	29	30	7	10	3	47.38
29	30	30	7	10	3	49.68

Table No. 6.3 Analysis of Variance (ANOVA) for the Quadratic Model

Sources of variations	Sum of squares	Degrees of freedom	Mean square	F-value	Probability
Model	4560.91	14	325.75	28.89	< 0.0001
Residual	188.75	15	12.58		
Error	81.42	5	16.28		
COR. Total	4749.21	29			

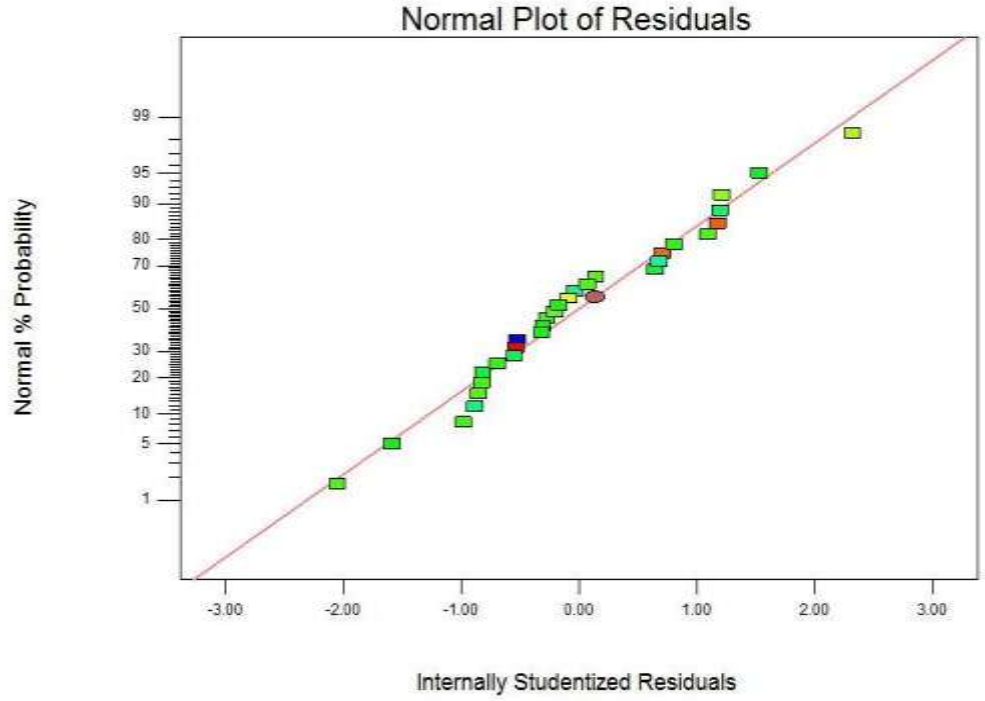
Standard Deviation = 3.55; CV % = 7.44; R^2 = 0.9603; Adj R^2 = 0.9232, Pred R^2 = 0.8451

Table No. 6.4 The Least Squares Fit and Significance of Regression Coefficients in Full Factorial Central Composite Design

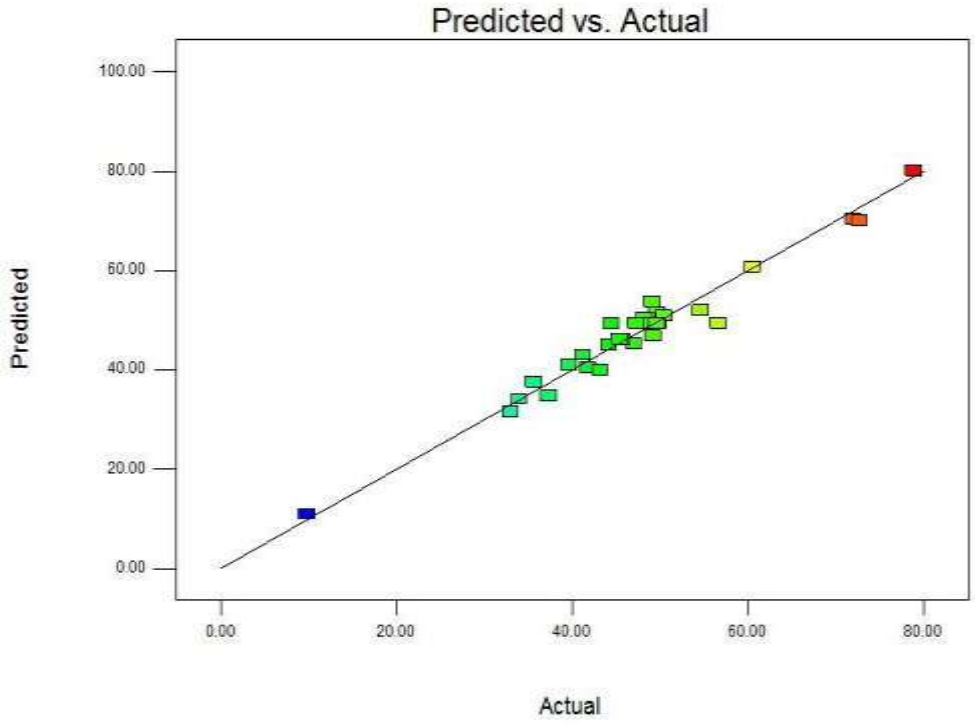
Model term	Regression Coefficient	Standard Error	p-value
Intercept	49.41	1.45	-
A-Temperature	7.06	0.72	< 0.0001
B-pH	0.086	0.72	0.9068
C-Enzyme Concentration	3.41	0.72	0.0003
D-Buffer Concentration	-6.06	0.72	< 0.0001
AB	1.1	0.89	0.2336
AC	3.28	0.89	0.0022
AD	-3.3	0.89	0.0021
BC	3.18	0.89	0.0027
BD	-0.32	0.89	0.7269
CD	-4.66	0.89	< 0.0001
A^2	-5.97	0.68	< 0.0001
B^2	0.51	0.68	0.4602
C^2	1.11	0.68	0.1206
D^2	2.17	0.68	0.0058

Table 6.5: Optimized reaction conditions based on selected criteria

Criteria	Temperature (°C)	pH	Enzyme Conc. (mg/ g oil)	Buffer Conc. (g/ g oil)	Response	Desirability
'Percentage of linoleic acid formed' is maximum, pH maximum, buffer and enzyme concentration minimum	33.62	7.5	6	2	61.04	0.884
Percentage of linoleic acid formed' is maximum, temp., pH and enzyme concentration minimum, buffer concentration maximum	25	6.5	6	4	46.11	0.879
'Percentage of linoleic acid formed' is maximum, pH and enzyme concentration minimum	32.8	6.5	6	2	54.84	0.867
'Percentage of linoleic acid formed' is maximum, temperature minimum, enzyme minimum and buffer maximum	25	6.5	6	4	46.11	0.852

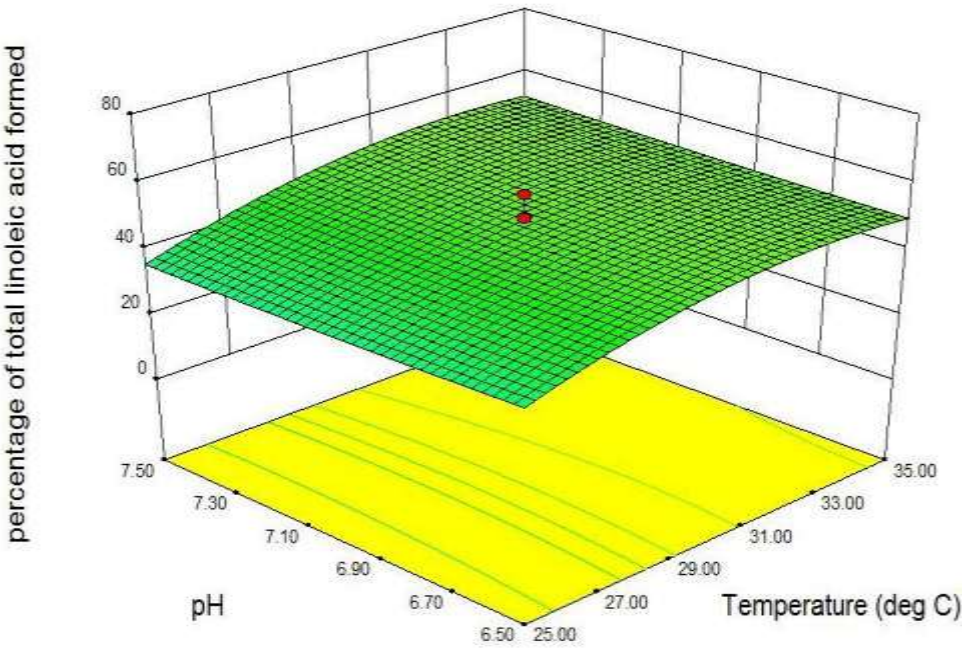


(a)



(b)

Fig.No. 6.1 Model validation plot (a) Normal % probability plot of internally studentized residuals for 'percentage of total linoleic acid formed (b) Relationship between the observed and the predicted 'percentage of total linoleic acid formed



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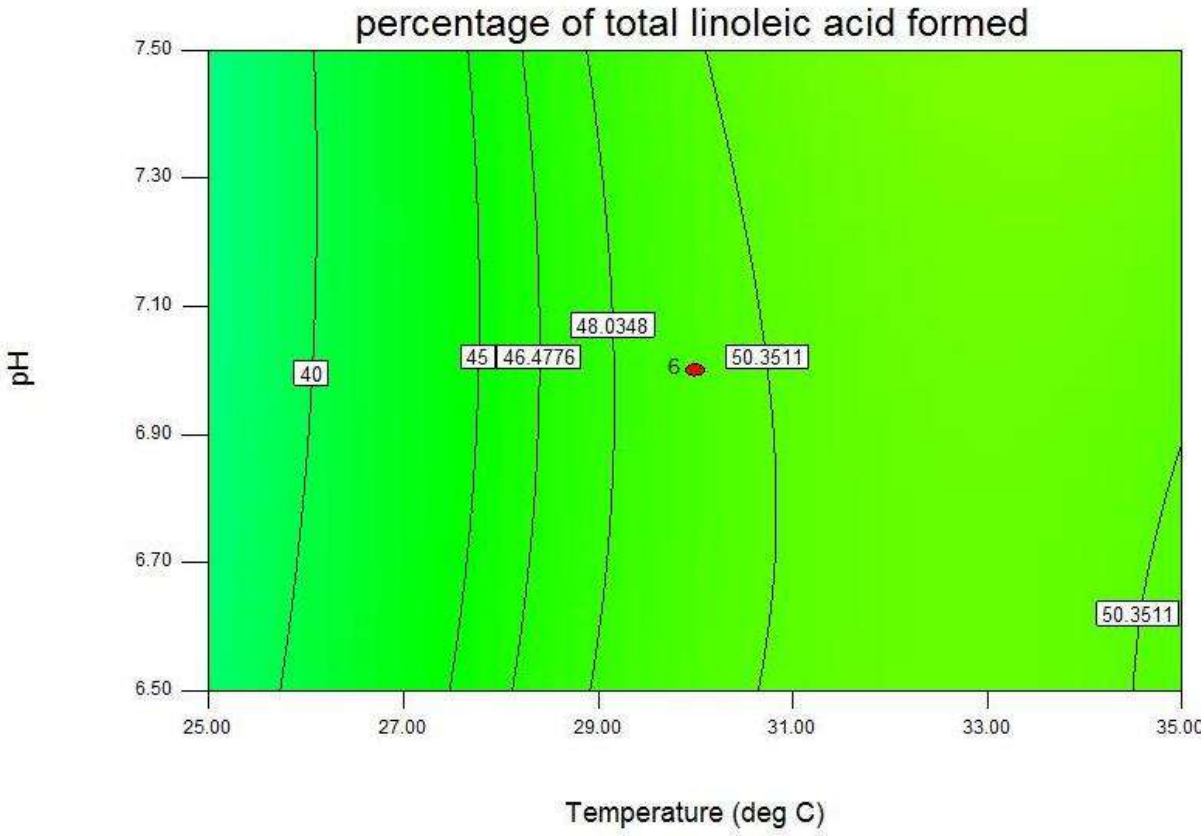
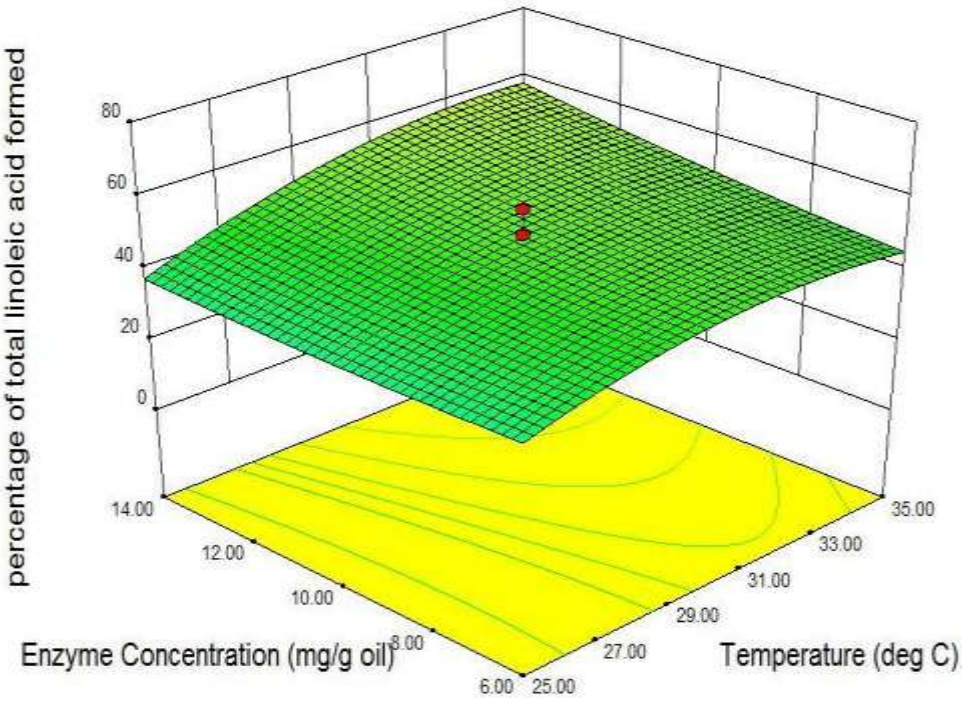


(b)

Fig.No. 6.2 Simultaneous interactive effect of temperature and pH on 'percentage of total linoleic acid formed'



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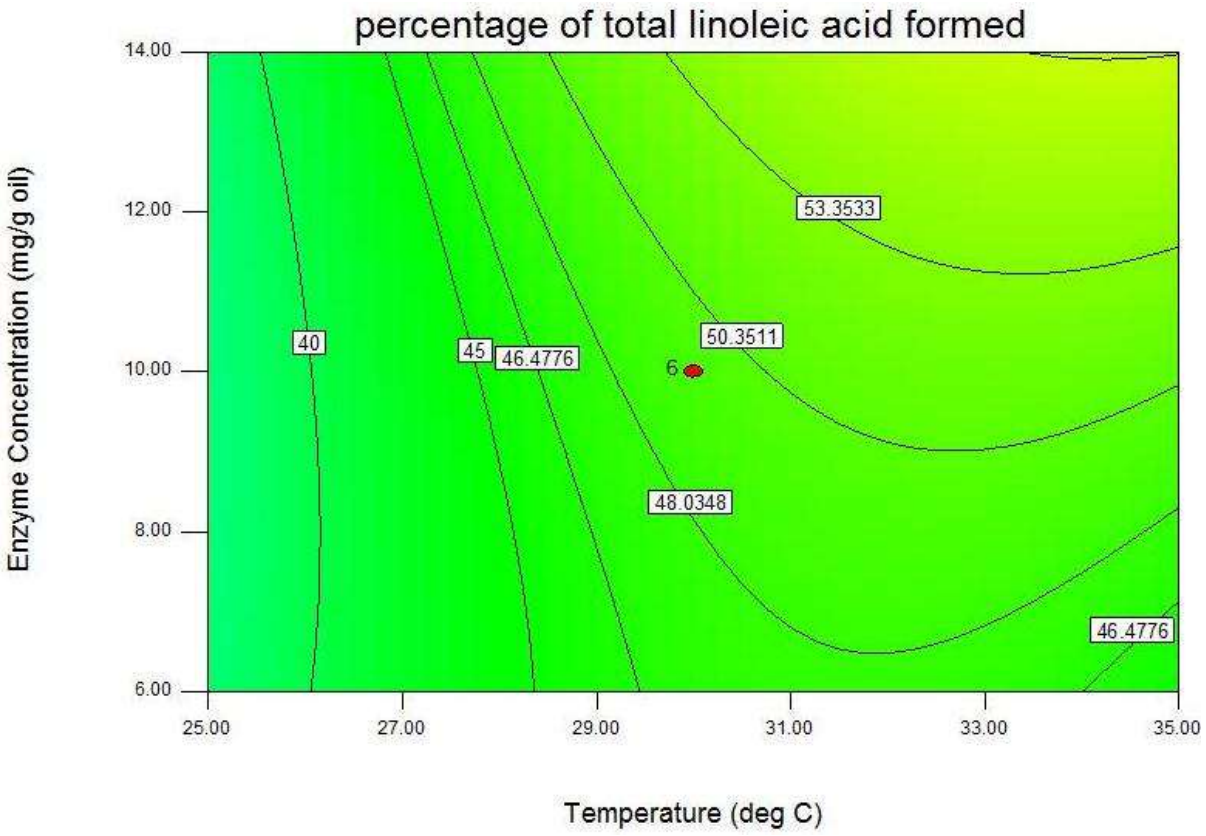

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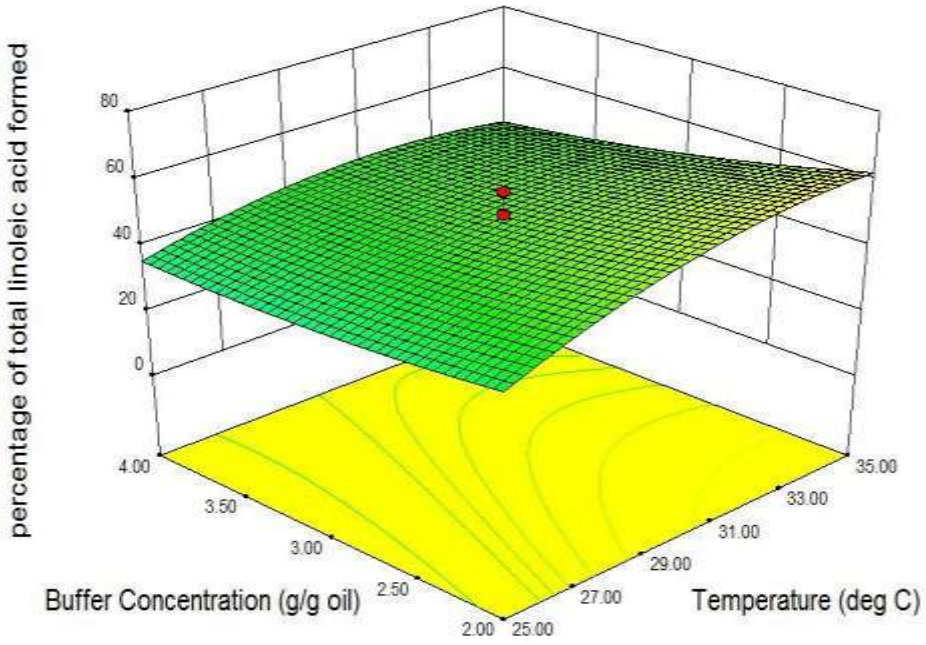

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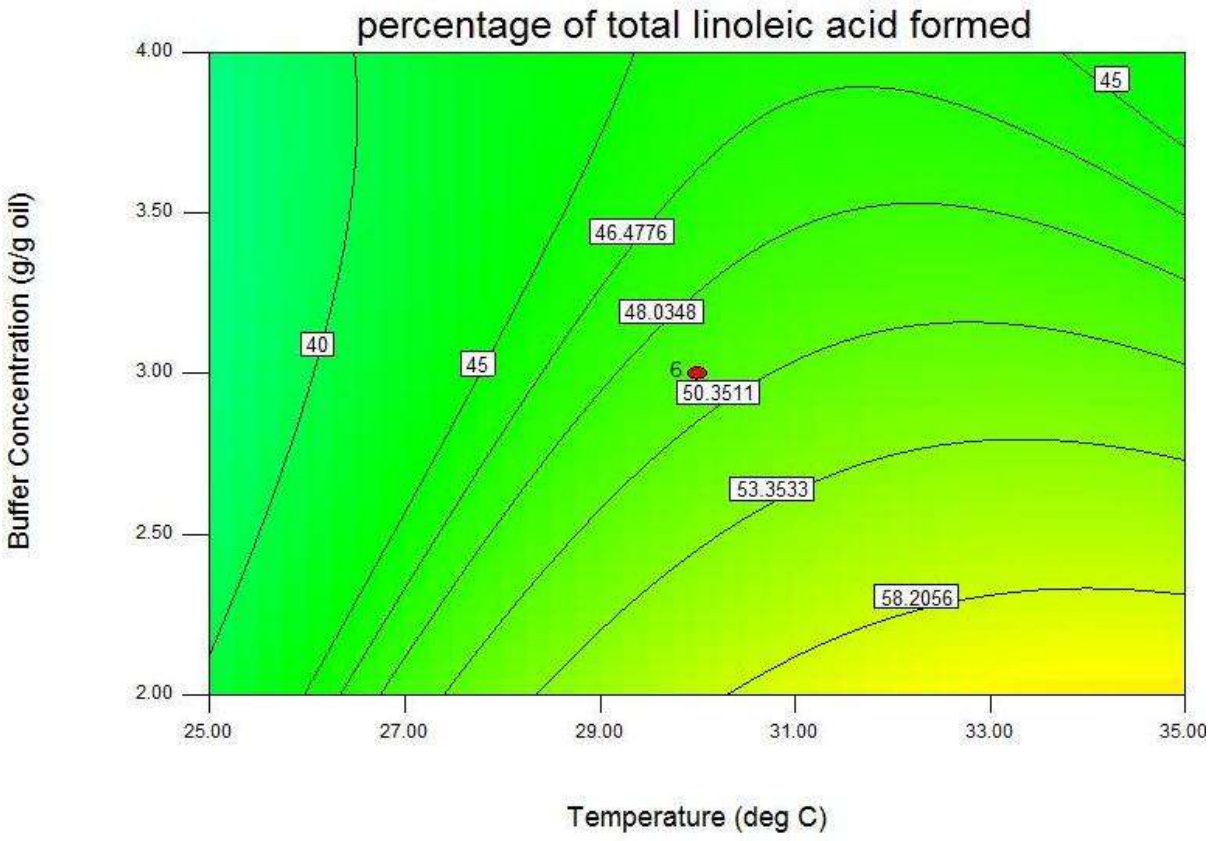
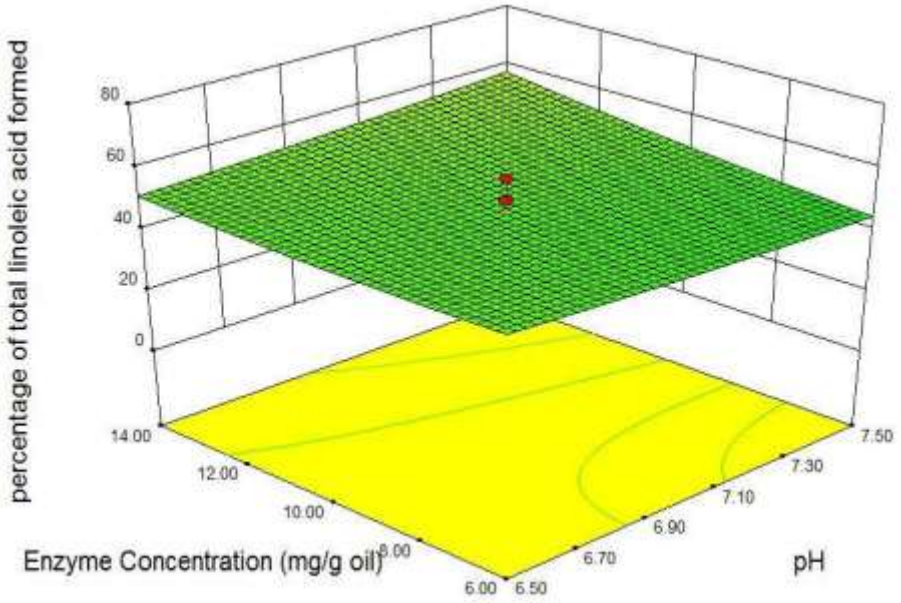


Fig.No. 6.4 Simultaneous interactive effect of temperature and buffer concentration on 'percentage of total linoleic acid formed'



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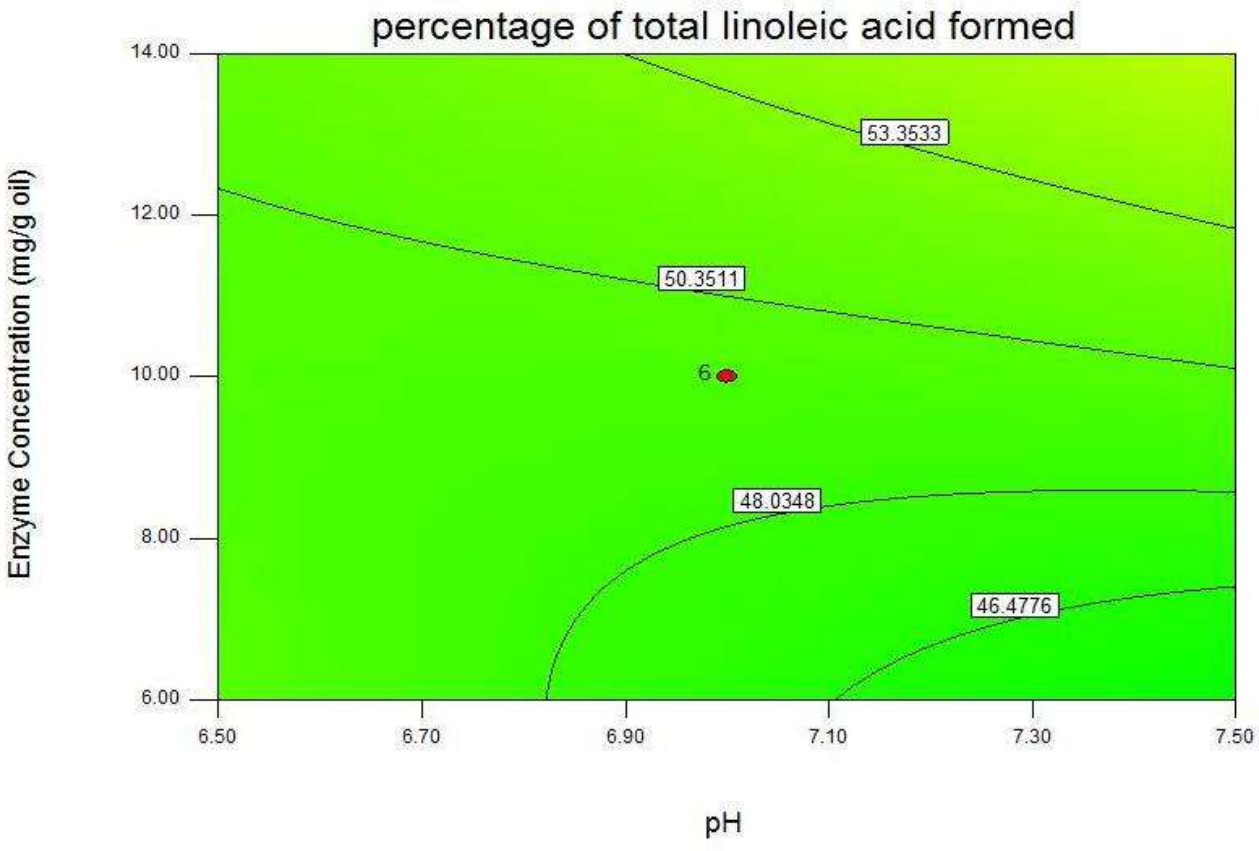
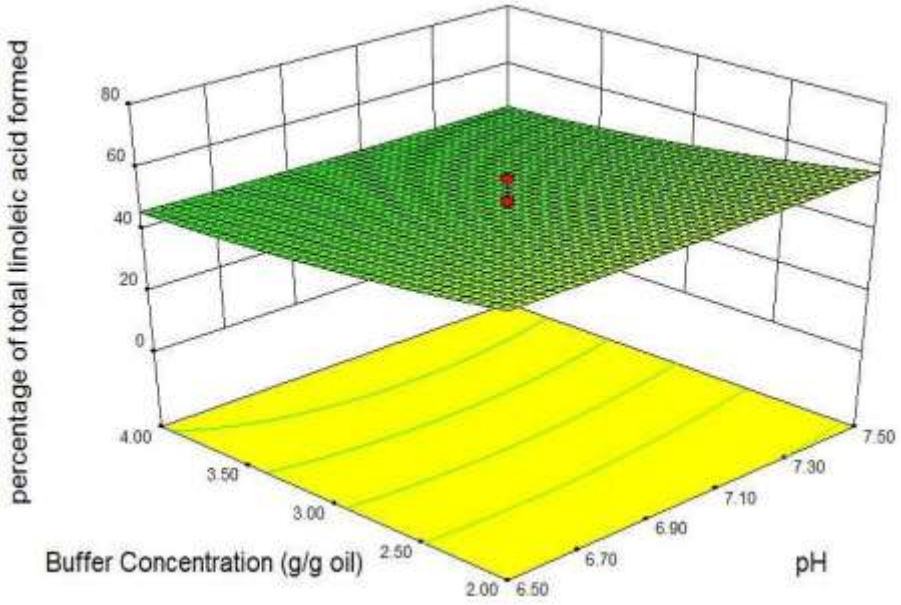


Fig.No. 6.5 Simultaneous interactive effect of pH and enzyme concentration on 'percentage of total linoleic acid formed'



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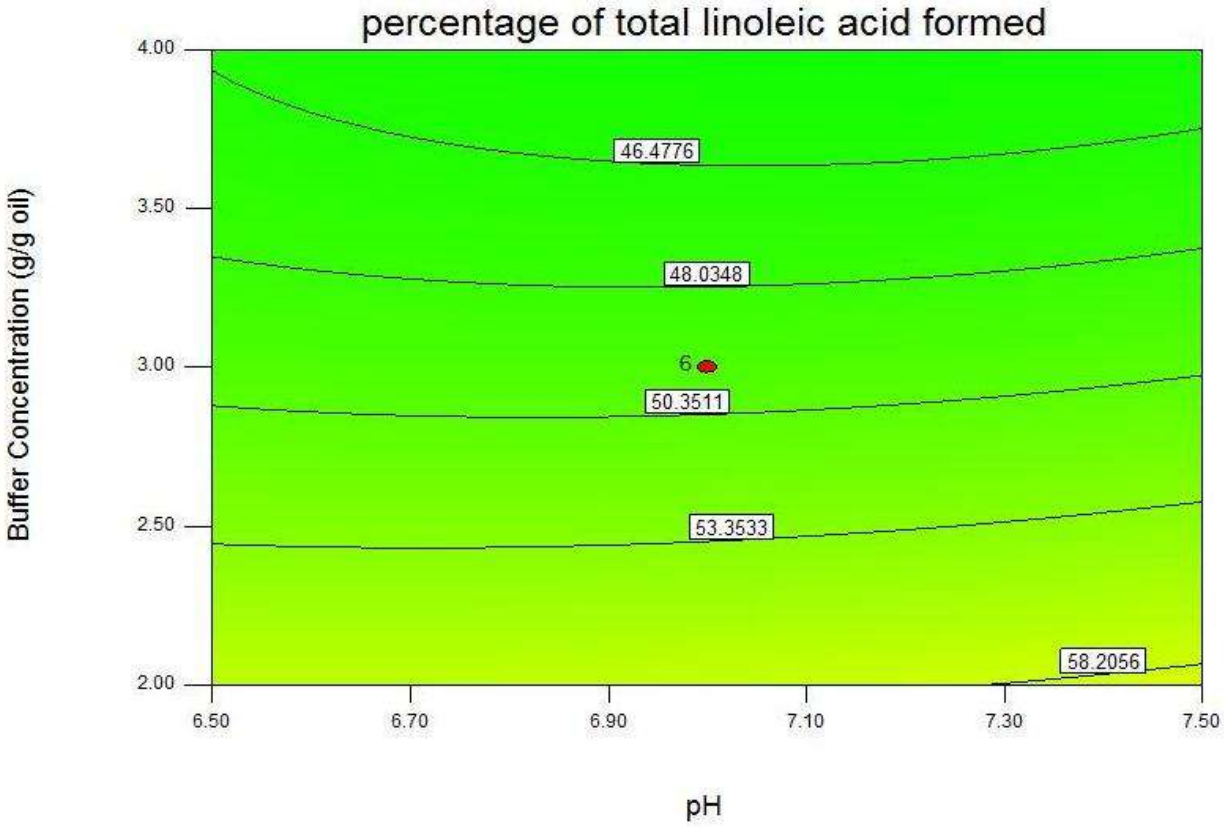
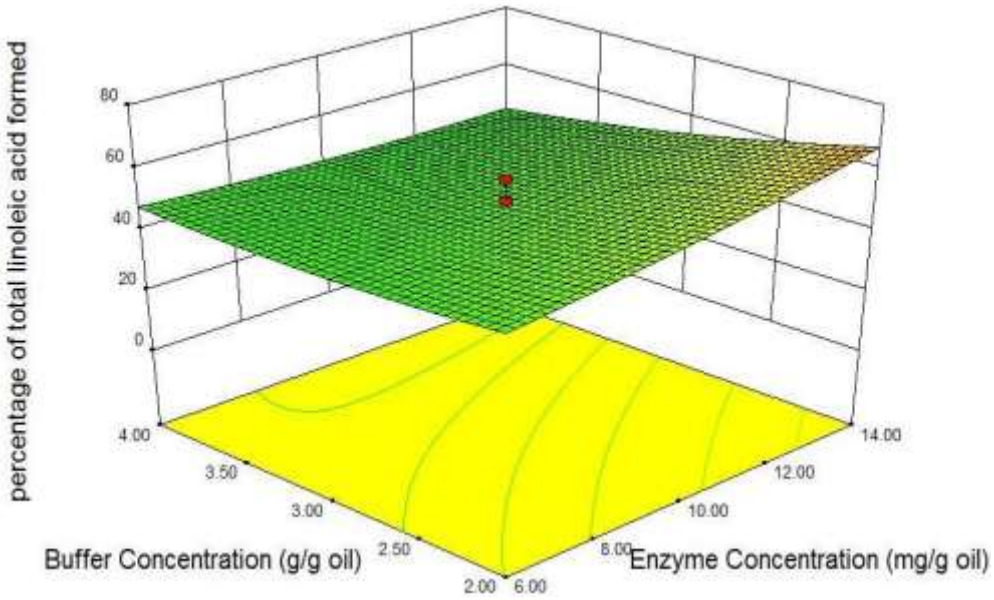


Fig.No. 6.6 Simultaneous interactive effect of pH and buffer concentration on 'percentage of total linoleic acid formed'



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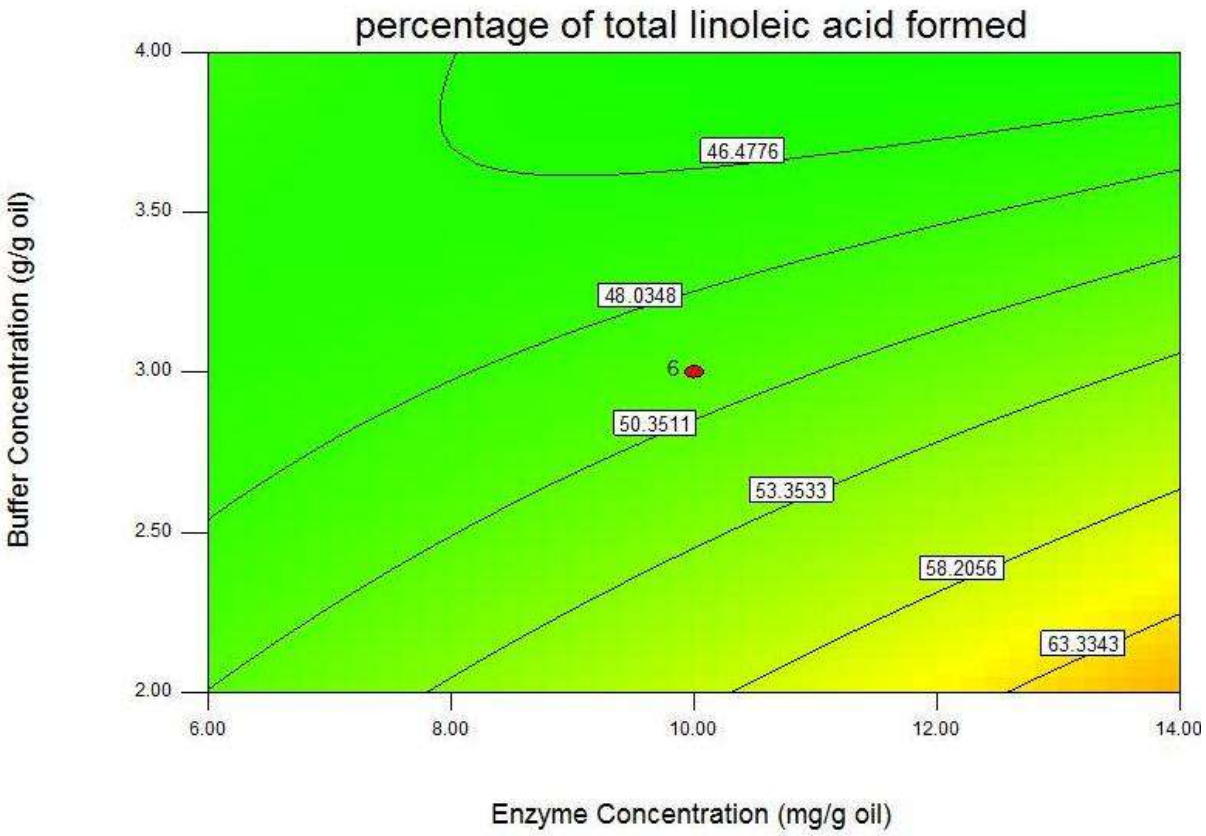

Fig.No. 6.7 Simultaneous interactive effect of enzyme concentration and buffer concentration on 'percentage of total linoleic acid formed'

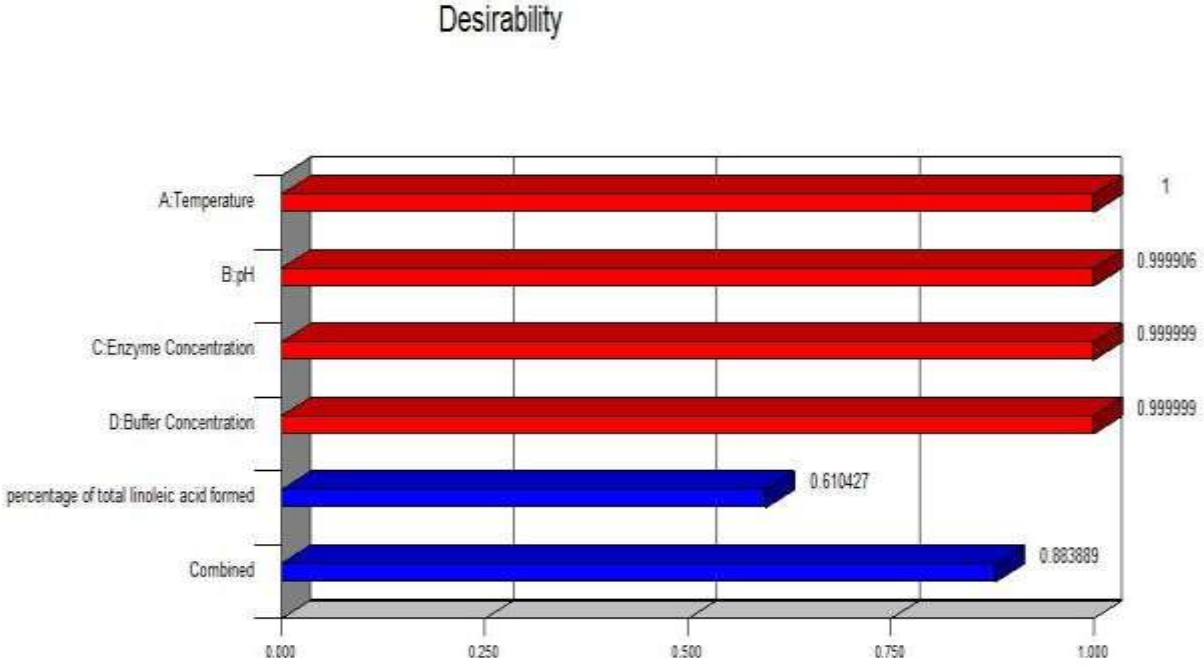

Fig. 6.8 Desirability plot for a typical optimization solution

Chapter 7

Kinetics Study of Oil Hydrolysis Catalysed by Lipase from *C. rugosa*

Chapter 7

Kinetics Study of Oil Hydrolysis Catalysed by Lipase from *C. rugosa*

Abstract

This chapter refers to the kinetic study performed on the hydrolysis of pumpkin and tobacco seed oil catalyzed by lipase from C.rugosa. The maximization of production of linoleic acid by hydrolysis of pumpkin and tobacco seed oil has been deliberated in detail in preceding chapters. Apart from optimization, kinetics study is essential as this focuses the effects of various process variables on total hydrolysis without highlighting on maximization of production of any specific fatty acid. The kinetics study indicated a first order kinetics followed by enzymatic hydrolysis of both oil samples.

7.1 Introduction

Kinetics study of enzymatic hydrolysis reaction measures the reaction rate and effects of changing the conditions of the reaction. The two most significant kinetic properties of an enzyme are how fast the enzyme becomes saturated with the specific substrate, and the maximum rate it can attain. Lipase catalyzed reactions take place at the interface for which the enzyme has to infiltrate the interface as a very first phase in the reaction [Tsai and Chang, 1993 and Al-Zuhair et al, 2003].The activity of lipases toward monomeric substrates in water is less significant but a higher activity is observed when the substrate can form either micellar aggregates or emulsions [Sarda and Desnuelle, 1958]. Though it is expected to increase the rate of reaction with an increase in the enzyme concentration, there would be an optimum enzyme concentration at which the interfacial area becomes saturated with the infiltrated enzyme. Beyond this concentration no increase in rate of reaction is observed [Al-Zuhair et al., 2004]. In order to optimize lipase activity in hydrolytic and cleaning applications, the kinetics of lipase action in O/W emulsions must be considered in detail.

7.2 The kinetic model

The basic steps of lipase catalyzed process like hydrolysis are [Jurado et al., 2006];

$$E + a_v \xrightarrow{k_a} E^* \xrightarrow{k_d} E + a_v \quad (7.1)$$

$$E^* + S \xrightarrow{k_1} E^*S \xrightarrow{k_{-1}} E^* + S \quad (7.2)$$

$$E^*S \xrightarrow{k_2} E^* + P \quad (7.3)$$

where, the terms E, E^* and E^*S represents free enzyme, active enzyme in adsorbed state and enzyme-substrate complex respectively. The terms $[a_v]$, S and P stand for vacant interfacial site concentration, substrate and product respectively. The terms k_a, k_d, k_1 and k_{-1} represents adsorption rate constant, desorption rate constant, rate constant in forward and backward step of formation of enzyme-substrate complex respectively. The rate of adsorption is given as;

$$- r_{ads} = k_a[E][a_v] - k_d[E^*] \quad (7.4)$$

As equilibrium is reached during adsorption,

$$-r_{ads} = 0$$

Concentration of active enzyme or $[E^*]$ is obtained as:

$$\text{Therefore, } [E^*] = \frac{k_a}{k_d}[E][a_v] = K_a[E][a_v] \quad (7.5)$$

The vacant interfacial site concentration is given as;

$$[a_v] = [E_T^*] - [E^*] \quad (7.6)$$

where, $[E_T^*]$ is total interfacial enzyme concentration. Combining Eqs. (7.5) and (7.6), the following expression of $[E^*]$ is attained

$$[E^*] = \frac{K_A E_T^*}{1 + K_A[E]} \quad (7.7)$$

Assuming concentration of intermediate i.e., enzyme-substrate complex to be unchanged with time, the following equation holds.

$$\frac{d[E*S]}{dt} = 0 \quad (7.8)$$

Combining Eqs. (7.2), (7.3) and (7.8), the following expression is obtained;

$$[E^*S] = \frac{[E*]S}{k_M} \quad (7.9)$$

$$\text{where, } k_M = \frac{k_{-1} + k_2}{k_1} \quad (7.9a)$$

Initial enzyme concentration $[E_o]$ and initial substrate concentration $[S_i]$ are given as:

$$[E_o] = [E^*S] + [E^*] + [E] \tag{7.10}$$

$$[S_o] = [E^*S] + [S] \tag{7.11}$$

The rate of reaction can now be expressed as,

$$v = \frac{dP}{dt} = k_2[E^*S] \tag{7.12}$$

Using Eq. (7.9), the expression of rate of reaction is obtained as;

$$v = \frac{k_2[E^*][S]}{k_M} \tag{7.12a}$$

Combining Eqs. (7.9) and (7.10), the expression of initial enzyme concentration is found as;

$$[E_o] = \frac{[E^*][S]}{k_M} + [E^*] + [E] \tag{7.13}$$

For high enzyme concentration, i.e., $K_A[E] >> 1$, from Eq. (7.7), the expression of $[E^*]$ is attained as;

$$[E^*] = [E_T^*] \tag{7.14}$$

Combining Eqs. (7.9) and (7.11), the following expression of [S] is obtained;

$$[S] = \frac{[s_o]}{\frac{[E^*]}{k_M}+1} \tag{7.15}$$

Combining Eqs. (7.12a) and (7.15), the expression of rate of reaction is obtained as;

$$v = \frac{k_2[E^*][s_o]}{k_M\{\frac{[E^*]}{k_M}+1\}} \tag{7.16}$$

By substituting $[E^*]$ by $[E_T^*]$ with Eq. (7.16),

$$v = k' [S_o] \tag{7.17}$$

where, apparent rate constant (K') can be expressed as,

$$k' = \frac{k_2}{\{\frac{k_M}{E_T^*} + 1\}} \quad (7.17a)$$

Initial rate of reaction (v_o) can be attained when analysis is based upon sample taken within a short course of time (20 minutes) from the beginning.

7.3 Results and Discussions

7.3.1 Hydrolysis of pumpkin seed oil using *Candida rugosa* lipase

The molecular weight of Candida rugosa lipase differs in the range of 57- 62 kDa [Sailas et al., 2000, de Maria et al., 2006a]. So, its average molecular weight is measured to be 60kDa i.e., 60,000 Da. In order to study kinetics, concentration of both oil (substrate) and enzyme were expressed on the basis of total volume of reaction mixture. The initial enzyme concentration (E_o) was varied from 7 to 49 mg i.e., from 1.944×10^{-3} mol/m^3 to 13.608×10^{-3} mol/ m^3. Initial substrate (oil) concentration (S_o) was varied from 125.86 mol/m^3 to 629.32 mol/ m^3. Speed of agitation and pH were kept constant at 1200 rpm and 7.5 respectively. Variation of initial rate of reaction (v_o) as a function of initial enzyme concentration (E_o) for different S_o has been shown in Fig 7.1. This figure indicates that for all values of S_o, v_o is increasing upto a certain value of E_o (9.720×10^{-3} mol/m^3) and then becomes nearly constant or a little decreasing. Therefore, this initial enzyme concentration is the optimum enzyme concentration under above-mentioned fixed conditions. Also, this figure shows, v_o of 23.917 mol/m^3.min at 9.720×10^{-3} mol/m^3 value of optimum initial enzyme concentration. It can be determined that with increasing S_o, v_o increases progressively at a fixed E_o.

Fig. 7.2 shows the variation of apparent rate constant (k') with E_o and it indicates that at E_o of 9.720×10^{-3} mol/m^3, k' becomes maximum and then almost constant. This means at this value of E_o, k_2 i.e., rate constant at Eq. (7.3) becomes maximum.

Temperature has an important effect on the initial rate of reaction. The variation of k' as a function of temperature is shown in Fig. 7.3 which clearly depicts that unlike ordinary catalyzed reactions, v_o first increases and then decreases with increasing temperature in enzymatic hydrolysis catalyzed by *C. rugosa* lipase. It can be seen from the graph that k' reaches to a maximum value (0.046 min^{-1}) at 35ºC. Then the value of k' rapidly falls to 0.029 min^{-1} at 45ºC. Denaturation of lipase at higher temperature may be the reason for this sudden fall of k' value. Therefore, 35ºC can be considered as an optimum temperature.

Fig 7.4 shows the variation of initial substrate concentration with initial rate of reaction. The figure clearly depicts a linear relationship between these two indicating a first order hydrolysis reaction catalysed by lipase from *C.rugosa.*

7.3.2 Hydrolysis of tobacco seed oil using *Candida rugosa* lipase

In this case, initial enzyme concentration was chosen in the range of 7 to 49 mg i.e., from 1.944×10^{-3} mol/m^3 to 13.608×10^{-3} mol/ m^3. Initial substrate (oil) concentration (So) was varied from 122.76 mol/ m^3 to 613.81 mol/ m^3. Speed of agitation and pH were kept constant at 1100 rpm and 7.0 respectively.

Fig. 7.5 shows the variation of initial rate of reaction with E_o keeping S_o as a parameter. This figure depicts that for all values of S_o, v_o is increasing upto a certain value of E_o (9.720×10^{-3} mol/m^3) and then becomes almost constant. At this optimum value of E_o, v_o is noted to be at 21.426 mol/m^3.min at S_o of 613.81 mol/m^3 and temperature 35ºC.

Fig. 7.6 indicates a variation in initial enzyme concentration as a function of k'. This figure shows that k' becomes maximum (0.043 min^{-1}) at E_o 9.720×10^{-3} mol/m^3. Fig. 7.7 represents the variation of apparent rate constant with temperature in tobacco seed oil hydrolysis catalyzed by *C.rugosa* lipase. This figure indicates that on increasing temperature from 25 to 45ºC, the rate constant first increases, extents maximum at 30ºC and then decreases abruptly from 35 to 45ºC. So, the optimum temperature was noted down at 30ºC at which k' is at 0.043 min^{-1}. Fig 7.8 indicates a linear relationship between initial rate of reaction and initial substrate concentration clearly indicating it as a first order hydrolysis reaction.

7.4 Conclusions

Lipase catalyzed hydrolysis of both pumpkin and tobacco seed oil follows first order kinetics as for both oil hydrolysis the initial rates of reaction are maximum upto a certain enzyme concentration and then becomes almost constant regardless of substrate concentration and also a linear relationship between substrate concentration verses initial velocity graph.

In case of pumpkin and tobacco seed oil hydrolysis, a maximum v_o of 23.917 mol/m^3.min and 21.426 mol/m^3.min is achieved at E_o of 9.720×10^{-3} mol/m^3. In other words optimum enzyme concentration is same for lipase catalyzed hydrolysis of both oil samples.

Thus, in case of pumpkin seed oil hydrolysis, at constant agitation speed of 1200 rpm, pH 7.5, v_o is attained at 23.917 mol/m^3.min at optimum enzyme concentration of 9.720×10^{-3}

mol/m^3, S_o 629.32 mol/m^3 and temperature 35ºC. Similarly, in case of tobacco seed oil hydrolysis, at constant agitation speed of 1100 rpm, pH 7.0, v_o is attained at 21.426 mol/m^3.min at optimum enzyme concentration of 9.720×10^{-3} mol/m^3, S_o 613.81 mol/m^3, and temperature 30ºC.

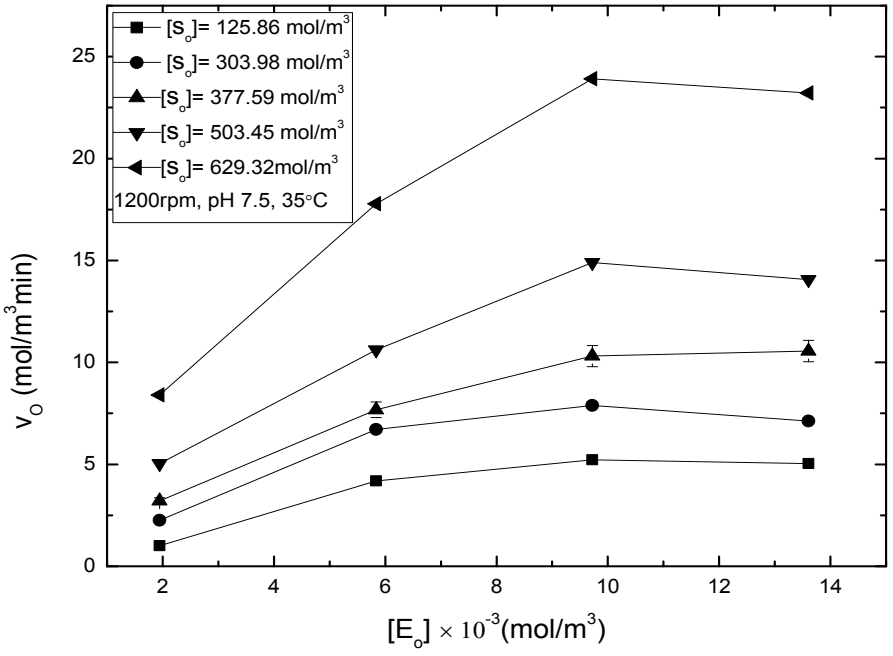

Fig. 7.1 Variation of initial rate of reaction with initial enzyme concentration keeping substrate concentration as a parameter in hydrolysis of pumpkin seed oil

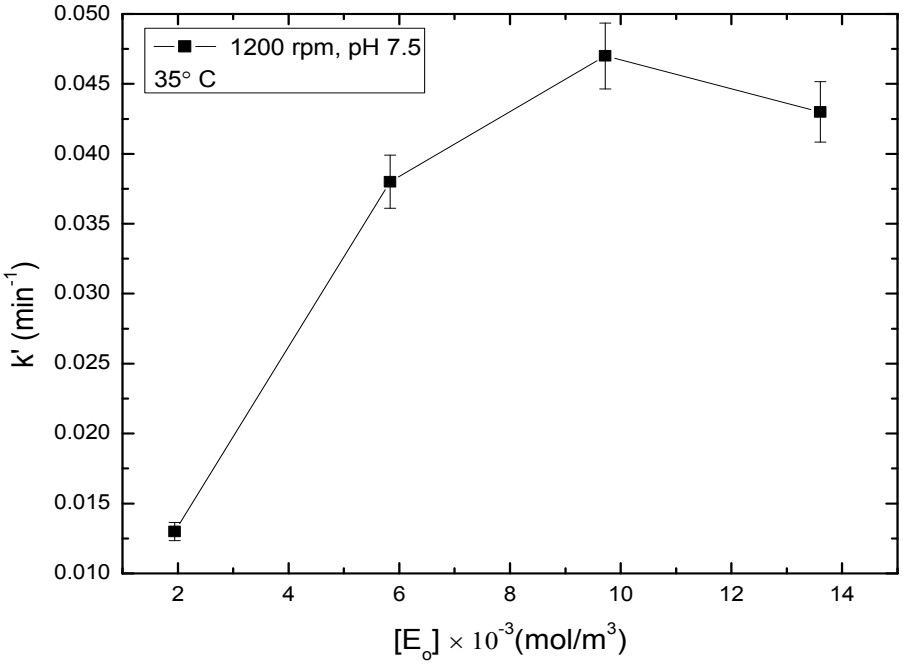

Fig. 7.2 Variation of apparent rate constant with initial enzyme concentration in hydrolysis of pumpkin seed oil

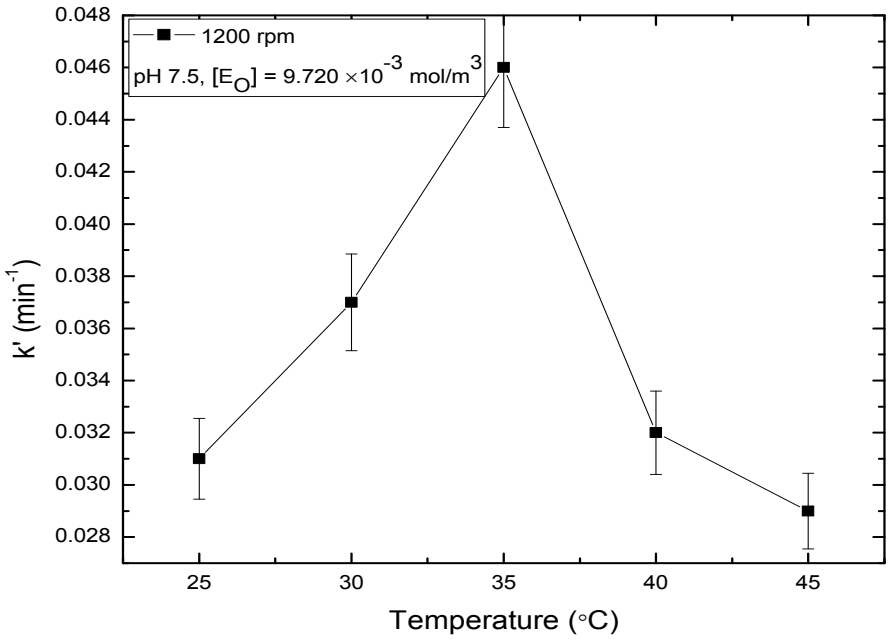

Fig. 7.3 Variation of apparent rate constant with temperature in hydrolysis of pumpkin seed oil

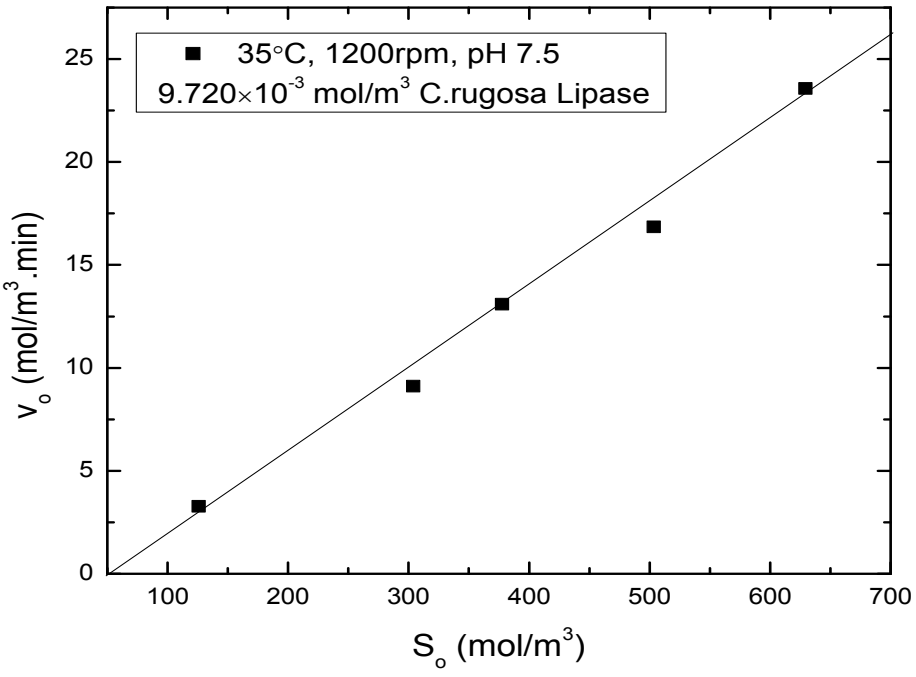

Fig. 7.4 Variation of initial rate of reaction with substrate concentration in hydrolysis of pumpkin seed oil

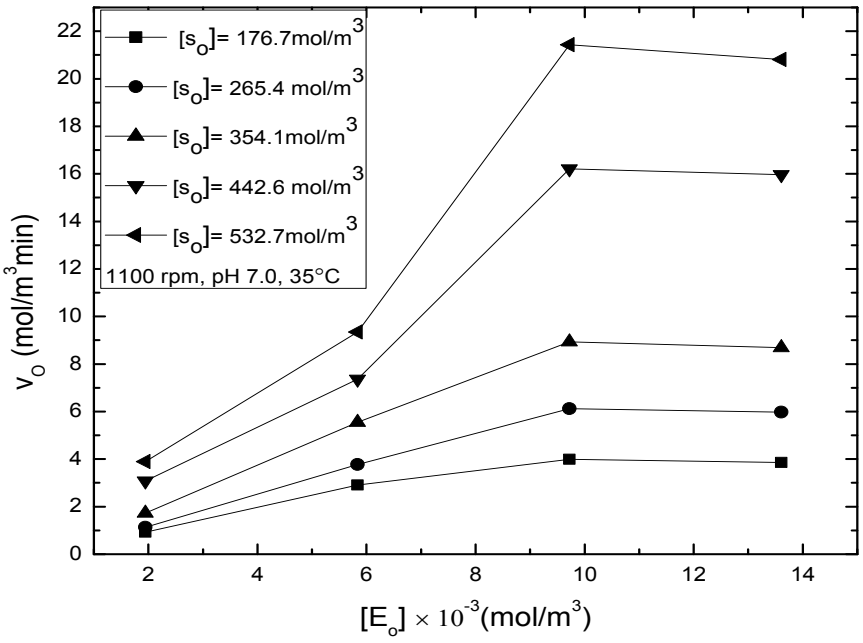

Fig. 7.5 Variation of initial rate of reaction with initial enzyme concentration keeping substrate concentration as a parameter in hydrolysis of tobacco seed oil

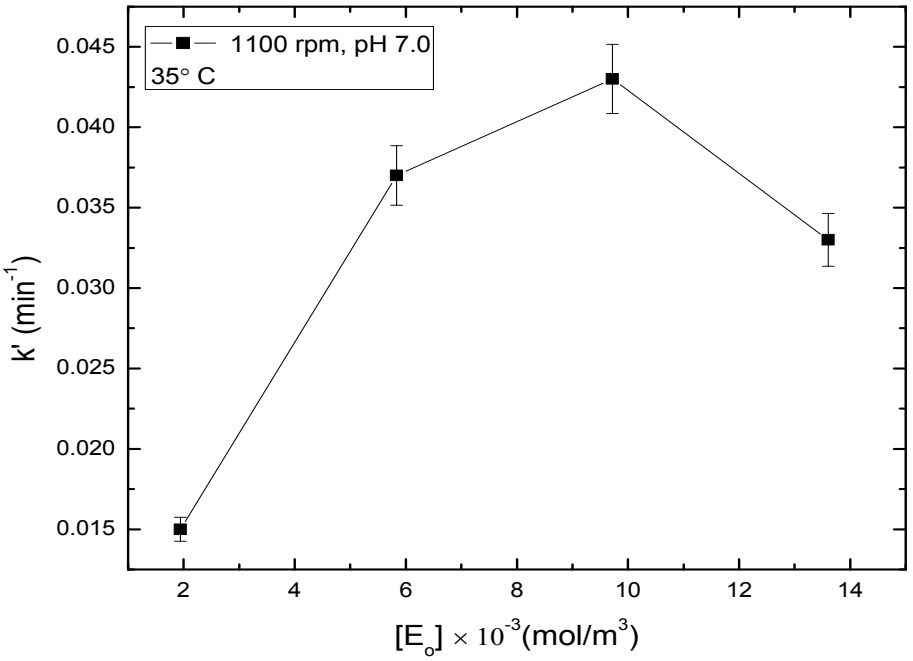

Fig. 7.6 Variation of apparent rate constant with initial enzyme concentration in hydrolysis of tobacco seed oil

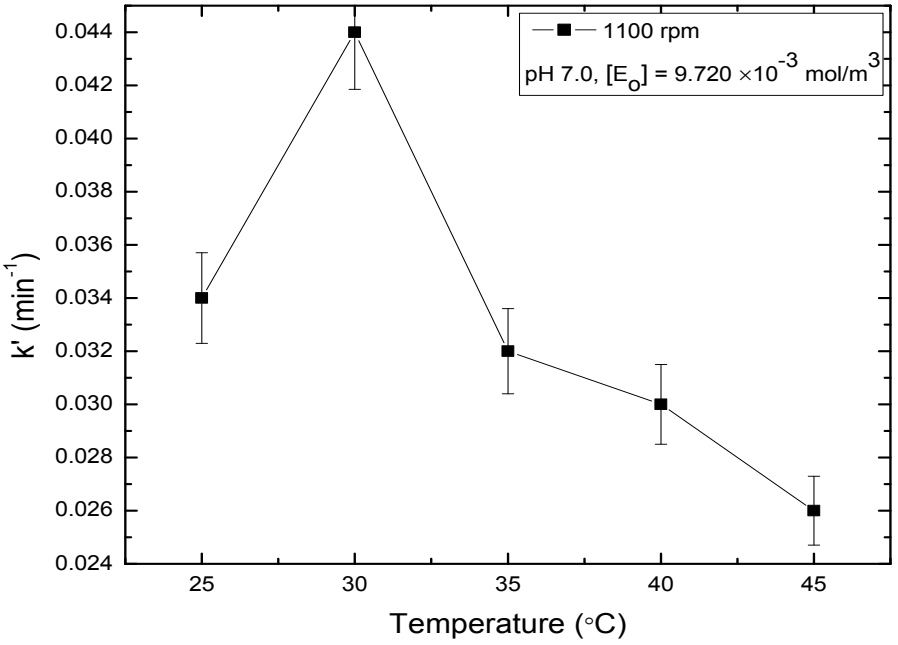

Fig. 7.7 Variation of apparent rate constant with temperature in hydrolysis of tobacco seed oil

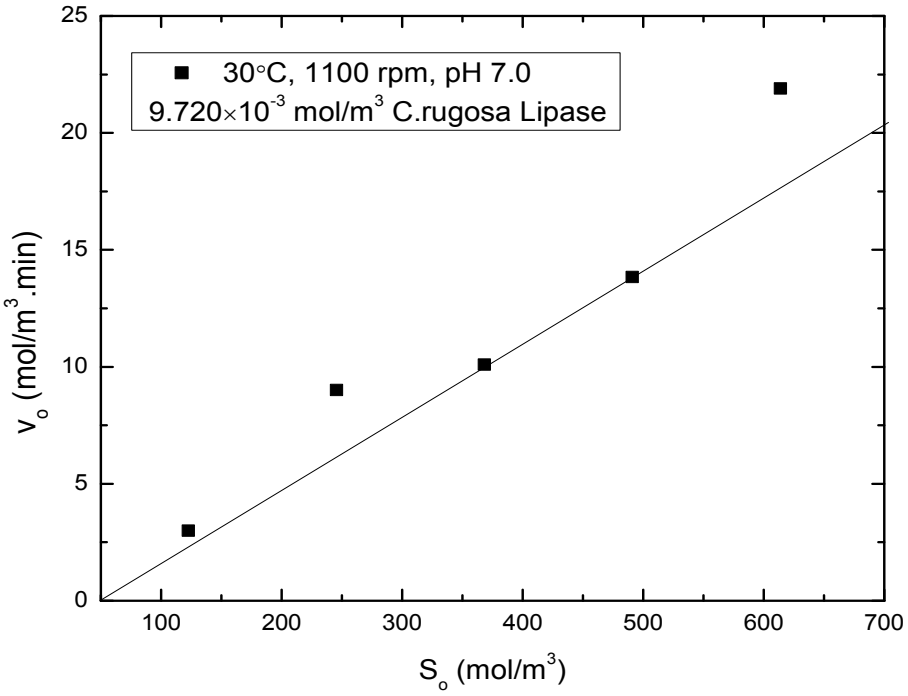

Fig. 7.8 Variation of initial rate of reaction with substrate concentration in hydrolysis of tobacco seed oil

Chapter 8

Conclusion & Future Perspective

Chapter 8

Conclusion & Future Perspective

Abstract

This chapter reviews the concluding comments from all previous chapters. Further, this chapter recommends the possibility for further work based on the observations, results and conclusions drawn from the present study and to get rid of some problems faced here.

8.1 Conclusion

Production of fatty acids by lipase catalyzed hydrolysis of oil has certain restrictions like denaturation of lipase by high speed of agitation or increased temperature or at greater pH or replacement of triacylglycerol and lipase from oil-water interface by more surface active reaction products like monoacylglycerol and fatty acids,. So, maximization of fatty acid production can be attained only at a combination of optimum values of respective process parameters. Therefore, this knowledge has been implemented in this study to obtain industrially important linoleic acid.

1. Two oil samples namely, pumpkin and tobacco seed oils were selected for hydrolysis catalyzed by *C. rugosa* lipase.
2. Single variable optimization method showed the effects of each parameter on the hydrolysis of both pumpkin and tobacco seed oils individually. Optimization was carried out with agitation speed, pH, temperature, buffer and enzyme concentration.
3. Optimum agitation speeds were observed at 1200 rpm and 1100 rpm for pumpkin and tobacco seed oil respectively. Optimum pH for pumpkin and tobacco seed oil was found to be 7.5 and 7.0 respectively. Optimum temperature was noted down at 35ºC for both oil samples. Similarly, buffer concentration was observed to be optimum at 1 g/g oil for both oils. Optimum value of enzyme concentration was monitored at 10 mg/g oil and 14 mg/g oil.
4. In case of pumpkin seed oil hydrolysis, optimization by RSM was carried out for four factors namely, temperature, pH, buffer and enzyme concentration. It indicated significant interaction between temperature and pH, temperature and enzyme concentration and pH and enzyme concentration. Optimum temperature, pH, buffer and enzyme concentration were found to be in range from 31 to 35ºC, 6.8 to 7.0, 9.0 to 10.0 mg/g oil and 2 to 2.3 g/g oil respectively.

5. Desirability for 'percentage of total linoleic acid formed' was calculated as 0.829 and combined desirability (D) as 0.939. The most desirable reaction condition for maximum production of 'percentage of total linoleic acid' (82.9%) was at temperature = 35°C, pH = 7.5, buffer concentration = 4 g/g oil and enzyme concentration = 10 mg/g oil.
6. For tobacco seed oil, significant interaction was observed for temperature with enzyme and buffer concentration. Also, significant interaction between pH & enzyme concentration and enzyme & buffer concentration was revealed by RSM.
7. Optimum temperature, buffer and enzyme concentration were found to be in range from 7.3 to 7.5, 3.0 to 4.0 g/g oil and 9.0 to 11.0 mg/g oil showing a much little amount of enzyme concentration and moderate pH condition needed for the production of linoleic acid.
8. Numerical optimization figured the overall optimization and resulted to 0.883 of combined desirability under optimum reaction conditions indicating a cost effective process by RSM.
9. Therefore, optimization by RSM proved effective results with higher production of linoleic acid at optimum reaction conditions in case of both pumpkin and tobacco seed oil as compared to single variable optimization method.
10. Kinetic study presented a first order kinetics in case of hydrolysis of both pumpkin and tobacco seed oil.
11. Thus, in case of pumpkin seed oil hydrolysis, at constant agitation speed of 1200 rpm, pH 7.5, v_o is attained at 23.917 mol/m3.min at optimum enzyme concentration of 9.720×10^{-3} mol/m3, S_o 629.32 mol/m^3 and temperature 35ºC. Similarly, in case of tobacco seed oil hydrolysis, at constant agitation speed of 1100 rpm, pH 7.0, v_o is attained at 21.426 mol/m^3.min at optimum enzyme concentration of 9.720×10^{-3} mol/m^3, S_o 613.81mol/m^3, and temperature 30ºC.

8.2 Perspective of future work

The restrictions of this process with their possible remedies have to be studied such that conversion to desired product can be improved further. Outlook of future work is mentioned below:

1. Search for a better lipase as catalyst can improve the production of linoleic acid from these two oil samples.

2. Studying the hydrolysis process with immobilized lipase and reusability of immobilized lipase can be studied further to enhance the production of desired product.
3. Comprehensive modeling of process allowing for different types of possible inhibition like substrate and product inhibition which remains pronounced in enzymatic hydrolysis.
4. More emphasize on kinetic study to improve the selectivity of product.
5. Detailed cost analysis of immobilized enzymatic hydrolysis process combined with membrane separation techniques to better know the simultaneous reaction and separation of products followed by optimization study can be performed.

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International Journal

- **Pandey, M., Sen, S., Singh, R. K., "***Enzymatic Hydrolysis of Non-Conventional Oil Resources: A Review***",** communicated to **'Bioresource Technology'.**
- **Pandey, M., Sen, S., Singh, R. K., "***Optimization of Enzymatic Hydrolysis of Tobacco seed Oil by lipase from C. rugosa***",** communicated to **'Bioresource Technology'.**

International Conference

- **Pandey, M., Sen, S., Singh, R. K., *"Enzymatic Hydrolysis of Non-Conventional Oil Resources: A Review"***, Biochemical Division, 65th Annual Session of IIChE, **CHEMCON – 2012** and **International Conference** on **"Sustainable Technologies for Energy and Environment in process Industrie**s**"**, NIT Jalandhar, Jalandhar, Punjab, India.

CURRICULUM VITAE

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M.Tech student, graduating in 2013 with major in Chemical Engineering from National Institute of Technology Rourkela with CGPA of 9.08/10. Areas of interest are Biochemical Engineering, Green Technology, Enzyme Technology and Microbiology. Had done internships in Enzyme production & characterization and Biodiesel production.

EDUCATIONAL QUALIFICATIONS

Master of Technology; National Institute of Technology, Rourkela (2011-2013)

Specialization: Chemical engineering

CGPA: 9.08

Bachelor of Technology; National Institute of Technology, Raipur (2006-2010)

Specialization: Biotechnology Engineering

Marks Percentage: 75.57%

12th Standard (CBSE- 2006)

Kendriya Vidyalaya Tuli, Nagaland

Marks Percentage: 74.2%

10th Standard (CBSE- 2004)

Kendriya Vidyalaya Tuli, Nagaland

Marks Percentage: 93.00%

TECHNICAL SKILLS

Enzyme Technology, Interfacial Science & Engineering

Software: MS Office, MINITAB, DESIGN EXPERT, Origin Pro, Chem Draw

OTHER ACADEMIC HIGHLIGHTS

Projects/ Training Undertaken

- *2008 June 15 days training in CSIR Laboratory (NEIST Jorhat, Assam) on "Study on the Production of Xylanase Enzyme by Trichoderma sp."*
- *2009 June 1 month training at KIMS Hospital & Research Centre, Dept. of Microbiology, Bangalore on ''Study of Techniques and Methodology performed in Biochemistry, Microbiology and Pathology".*
- *2009-2010 Project on "Characterization of Enzyme Papain isolated from Carica papaya".*
- *Recently, carrying out project on "Lipase catalyzed hydrolysis of Non-conventional Oil Resources: Kinetics & Optimization Study"*

Publications/ Conferences

- ***Pandey, M., Sen, S., Singh, R. K.*** *"Enzymatic Hydrolysis of Non-conventional Oil Resources: A Review", Communicated to* ***Bioresource Technology****.*
- ***Pandey, M., Sen, S., Singh, R. K.*** *"Enzymatic Hydrolysis of Pumpkin seed oil- Experimental design & Optimization of Parameters using Response Surface Methodology", Communicated to* ***Bioresource Technology****.*
- ***Pandey, M., Sen, S., Singh, R. K.*** *"Enzymatic Hydrolysis of Non-conventional Oil Resources: A Review", Biochemical Division, 65th Annual Session of IIChE,* ***Chemcon – 2012*** *and* ***International Conference*** *on* ***"Sustainable Technologies for Energy and Environment in Process Industries"****, NIT Jalandhar, Jalandhar, Punjab, India*

Scholastic Achievements

- Qualified GATE with 88.8 percentile in year 2011.